METABOLIC MECHANISMS OF THE FAT MASS AND OBESITY-ASSOCIATED (FTO) GENE

By

JESSICA DANAHER

Masters of Dietetics, APD

BSc Hon (Nutritional Therapy)

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy

Exercise Metabolism Unit

Centre for Chronic Disease Prevention and Management

College of Health and Biomedicine

Victoria University

Melbourne, Australia

Submitted December 2016

ABSTRACT

The modern obesogenic lifestyle encompasses an environment that promotes weight gain in the form of body fat accumulation. Genetic variations can predispose some individuals to be more susceptible to developing obesity in a similar environment (Hainer et al. 2000; Maes et al. 1997; Mustelin et al. 2009). As the prevalence of obesity increases worldwide, and becomes a substantial socioeconomic issue, the need for research that deepens our understanding of the underlying genetic influences on complex regulatory mechanisms governing energy homeostasis becomes increasingly necessary. The fat mass and obesity-associated (FTO) gene has been strongly linked to an increased obesity risk through numerous genome wide association (GWA) studies over the past decade. However, information regarding FTO's peripheral influences, specifically on skeletal muscle metabolism, is limited. The broad aim of this dissertation was to identify metabolic differences between risk allele and non-risk variants of FTO, and to determine the impact of physical stress (in the form of exercise) on FTO expression and function. Thus, the experiments presented in this dissertation were designed to investigate whether differences across genotype alleles of the FTO rs9939609 (T>A) polymorphism existed for metabolic flexibility in response to a nutritional challenge, and for metabolic profiles, FTO expression and FTO function in skeletal muscle following acute exercise stimuli.

Metabolic flexibility refers to the ability of the muscle to effectively switch between substrates supplying energy based on availability. Whilst FTO expression has been linked to peripheral defects of glucose and lipid metabolism in the muscle, it is unknown whether risk variants of FTO are associated with impairments to metabolic

flexibility. Thus, the study presented in Chapter 4 investigated whether differences in metabolic flexibility existed between allelic variants of the FTO rs9939609 polymorphism following an oral glucose load (OGL) challenge. The findings of this study support a previously established link between an overweight body mass index (BMI), slower metabolic flexibility and insulin resistance. However, when separating participants based on FTO genotype, no differences in metabolic flexibility existed. This finding could suggest that risk variants of FTO may not contribute to obesity predisposition via metabolic mechanisms. However, it is possible that the methodology selected to examine energy flux in this investigation (respiratory gas exchange analysis) may have not been sensitive enough to detect potential differences between FTO genotypes, or that the metabolic stimulus produced by an OGL may have been insufficient to influence FTO function.

Exercise stress imposes opposing effects on muscle metabolism compared to dietary exposure and was used as an alternate metabolic stimulus for further investigations on FTO's peripheral influences. Two acute bouts of isocaloric exercise (high and low intensity) were used in Chapter 5 to elicit intensity dependent metabolic disturbances in the skeletal muscle, and determine whether a specific skeletal muscle metabolic profile existed between FTO genotypes. This was the first human investigation (to the author's knowledge) to explore snapshots of metabolism, via skeletal muscle samples analysed using metabolomics technology, to profile genotype-associated responses to acute physiological stimuli. It was proposed that variations in metabolic profiles would exist between allelic homologues of FTO, and that these differences might provide important clues as to the metabolic mechanisms of FTO. Skeletal muscle glucose was the only metabolite found to consistently differ across genotypes in

response to exercise. Individuals homozygous for the risk A-allele (AA genotypes) had a significantly greater elevation of muscle glucose than that of individuals homozygous for the non-risk allele (TT genotypes), in the early stages following both high and low intensity exercise. Additionally, a trend for higher muscle fumarate/succinate was observed for genotypes encompassing the risk A-allele (AA and AT) compared to the TT genotype at 10 minutes following high intensity exercise. These findings may be indicative of potential metabolic pathways and mechanisms by which FTO risk variants influence obesity predisposition.

There is a paucity of research concerning FTO gene and protein expression, as well as the demethylation function of FTO, in peripheral tissues. Prior to this dissertation, no human investigation had used exercise as a metabolic stimulus to explore the function of FTO. Thus, the purpose of the study presented in Chapter 6 was to elicit exercise intensity dependent effects on FTO expression and function, and to determine whether genotypic differences in these variables existed in skeletal muscle. A novel finding from this research was an acute up regulation of skeletal muscle FTO mRNA in the early stages following high intensity exercise, with AA genotypes showing a trend (non-significant) for greater FTO mRNA expression in the early stages following high intensity exercise compared to TT genotypes. FTO mRNA expression was positively correlated with skeletal muscle glucose in AA genotypes during the high intensity exercise trial, with no relationship between these variables detected for AT or TT genotypes. This may provide a clue to a possible mechanism by which FTO risk variants could impact an individual's predisposition to obesity or metabolic complications. Neither high or low intensity exercise, nor genotype, was found to influence FTO protein expression levels. Of interest, only the non-risk variant showed

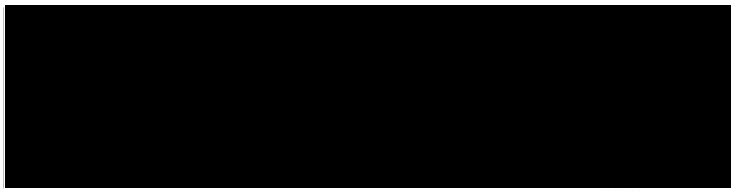
a significantly greater demethylation of m^6A on RNA (a marker of FTO function) from baseline following low intensity exercise. Thus, low intensity exercise may suppress the demethylation function of FTO in individuals carrying the risk A-allele, whilst facilitating demethylation in TT genotypes.

The investigation presented in Chapter 7 was designed as a pilot study to develop and optimise metabolomics specific methodologies in plasma, prior to this technology being used to analyse skeletal muscle tissue in Chapter 5. This investigation utilised an untargeted metabolomics approach to examine the metabolic perturbations in plasma that occur in response to two workload matched supramaximal low volume high intensity exercise trials. The data obtained showed that an untargeted metabolomics approach can provide researchers with the ability to examine a wide range of metabolic interactions and potential pathways (not observed with targeted approaches), and can be used to monitor intensity-dependent changes in multiple metabolic pathways following exercise. Additionally, this study provided support for the beneficial impact of supramaximal exercise on total cholesterol and other lipids, which may have positive health implications.

The studies presented in this dissertation contribute to a further understanding of the role of FTO in skeletal muscle. They show acute dietary glucose exposure to have no obvious influence on FTO, whilst acute exercise was found to have some modulating effect on FTO (an increased mRNA content). The mechanisms influencing the association between muscle glucose and FTO mRNA in AA genotypes following exercise are yet to be determined, providing a pathway for future research that explores the peripheral roles of FTO. Furthermore, research that explores the influence of repetitive exercise on FTO outcomes is also warranted.

DECLARATION

I, Jessica Danaher, declare that the PhD thesis entitled "Metabolic Mechanisms of the Fat Mass and Obesity-Associated (FTO) Gene" is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work.



Jessica Danaher

December 2016

ACKNOWLEDGEMENTS

Carrying out the requisite work was, undoubtedly, the most arduous task I have ever undertaken. However, one of the joys of having completed this thesis is looking back at everyone who has helped and supported me along the way.

Firstly, I would like to express my deepest appreciation to the one hundred and fifty-seven individuals who dedicated their time, personal information, blood, and in some cases skeletal muscle, to my projects. Without you this thesis would not exist. The highlight of my PhD was getting to know you all, even if it was for a short while, and at times being informed that I had inspired you to achieve an educational or health related goal. Thank you.

I would like to thank both of my supervisors, Dr Christos Stathis and Dr Matt Cooke, for their support throughout my PhD, keeping me going when times were tough, offering invaluable advice, and putting up with my relentless desire to complete a Doctorate and a Masters of Dietetics simultaneously (despite a few meltdowns).

Chris Stathis – It was you who convinced me to join the Exercise Metabolism Unit as part of my Summer Research Scholarship, and as such it is you I thank for putting me on the path that I am on today.

Matt Cooke - It was your visions that laid out the foundations of my Doctorate, and I thank you for encouraging me to explore the novel and unknown. Without your guidance, this thesis would not have been possible.

I would also like to thank the co-author of my 7th chapter, Dr Tracey Gerber, for her willingness to help, sense of humour and mentoring. I owe my knowledge on how to successfully run clinical trials and navigate in the lab to you.

Dr Mitch Anderson, thank you for always bringing positive vibes to my trials, your enthusiastic demeanour kept my muscle biopsy participants at ease and my work fun. Ms Charlotte Giuliani, I will always be thankful for your selflessness and willingness to provide me with Western Blotting advice, without you those damn blots would have sent me to the Psych Ward.

I would like to share my sincere gratitude to the extremely helpful employees of Metabolomics Australia at the University of Melbourne, in particularly Dr Daniel Dias who taught me almost everything I know regarding metabolomics. Dr Mark Wellard you were invaluable when it came to metabolomics result interpretation. Also, to all the laboratory technicians across Victoria University campuses – Thank you for being so prompt and fixing any technical issues that had me wanting to pull my hair out. Western Centre of Health, Research and Education students and staff, thank you for some great memories, lunch time chats and motivation.

My sincerest appreciation to Alexander Lamb, for your unwavering support and belief in me during the most challenging period of my life to date. To my closest gal pals, Alex Taylor and Chloe Walker – thank you both for making our wine nights a “safe place” to debrief. And lastly, to my family, particularly my parents Ken and Raelene Danaher, thank you for your love, support, and unwavering belief in me. Without you, I would not be the person I am today.

LIST OF PUBLICATIONS

The following publications have resulted for the work undertaken in this thesis:

Peer reviewed article:

Danaher, J, Gerber, T, Wellard, RM, Stathis, CG, & Cooke, MB 2016, 'The use of metabolomics to monitor simultaneous changes in metabolic variables following supramaximal low volume high intensity exercise', *Metabolomics*, vol. 12, no. 7, pp. 1-13.

Conference Abstracts:

Danaher, J, Gerber, T, Stathis, CG & Cooke, MB 2015, 'Metabolic responses to supramaximal low volume high intensity exercise: A metabolomics approach', *Medicine and Science in Sports and Exercise*, vol. 47, pp. 447.

Cooke, MB, **Danaher, J**, Greenwood, M & Stathis, CG 2015, 'The effect of exercise on muscle metabolism between FTO gene variants: A metabolomics approach', *Medicine and Science in Sports and Exercise*, vol. 47, pp. 446.

Cooke, MB, **Danaher, J** & Stathis, CG 2014, 'The common rs9939609 gene variant for fat mass and obesity associated gene (FTO) and metabolic flexibility', *Medicine and Science in Sports and Exercise*, vol. 46, pp. 625.

LIST OF PRESENTATIONS

Conference presentations resulting from the work undertaken in this thesis are listed:

Title of Presentation	Authors	Forum
The Common rs9939609 Gene Variant for Fat Mass and Obesity-Associated Gene FTO and Metabolic Flexibility	Cooke, MB **Danaher, J** Stathis, CG	American College of Sports Medicine (ACSM) 61st Annual Conference, 27th May – 31st May 2014, Florida, America. *Poster Presentation.*
The Common rs9939609 Gene Variant for Fat Mass and Obesity-Associated Gene FTO and Metabolic Flexibility	**Danaher, J** Cooke, MB Stathis, CG	Victoria University Health and Biomedicine Postgraduate Research Conference, 2nd October 2014, Victoria, Australia. *Oral Presentation.*
The Common rs9939609 Gene Variant for Fat Mass and Obesity-Associated Gene FTO and Metabolic Flexibility	**Danaher, J** Cooke, MB Stathis, CG	Victoria University/University of Texas El Paso, Health Research Symposium, 16th October 2014, Victoria, Australia. *Oral Presentation.*
Metabolic Responses To Supramaximal Low Volume High Intensity Exercise: A Metabolomics Approach	**Danaher, J** Stathis, CG Cooke, MB	American College of Sports Medicine (ACSM) 62nd Annual Conference, 27th May – 30th May 2015, San Diego, America. *Poster Presentation.*
The Effect of Exercise on Muscle Metabolism Between FTO Gene Variants: A Metabolomics Approach	Cooke, MB **Danaher, J** Greenwood, M Stathis, CG	American College of Sports Medicine (ACSM) 62nd Annual Conference, 27th May – 30th May 2015, San Diego, America. *Poster Presentation.*
A FTO Gene Variant and BMI Comparison of Resting Metabolism and Metabolic Flexibility in Males and Females	**Danaher, J** Cooke, MB Stathis, CG	Dietitians Association of Australia (DAA) 33rd National Conference, 19th May – 21st May 2016, Melbourne, Australia. *Poster Presentation.*

Please note: The author who presented this work has been underlined.

VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

TABLE OF CONTENTS

LIST OF FIGURES

Page

Page

Page

Page

LIST OF TABLES

Page

Page

LIST OF ABBREVIATIONS

°C	Degrees Celsius
Δ	Delta
2D	Two dimensional
2OG	2-oxoglutarate
3-meT	3-methylthymine
3-meU	3-methyluridine
ACTB	ß-Actin
Ala	Alanine
AlkB	Alkylatin B
ALKBH5	AlkB family member 5 RNA demethylase
AMDIS	Automated mass spectral deconvolution and identification system
AMP	Adenosine-5-monophosphate
amu	Atomic mass units
ANCOVA	Analysis of covariance
ANOVA	Analysis of variance
Arg	Arginine
Asn	Asparagine
Asp	Aspartate
ATP	Adenosine triphosphate
AUC	Area under the curve
BMI	Body mass index
BOLD	Blood oxygen level dependent
BSA	Bovine serum albumin
CO_2	Carbon dioxide
CON	Continuous exercise
C_T	Cycle threshold
CVD	Cardiovascular disease
DEXA	Duel energy x-ray absorptiometry
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
ECL	Enhanced chemiluminescence
EGTA	Ethylene glycol tetraacetic acid
EHIC	Euglycemic hyperinsulinemic clamp

EI	Electron ionization
ELISA	Enzyme-linked immunosorbent assay
ETC	Electron transport chain
eV	Electron volt
F	Female
FASN	Fatty acid synthase
Fe^{2+}	Ferrous
FTO	Fat mass and obesity-associated gene
Fto	Fat mass and obesity-associated gene protein (mouse models)
G	Gravity/acceleration due to gravity
g	Grams
G6PC	Glucose-6-phosphatase
G6PD	Glucose-6-phosphate dehydrogenase
GABA	Gamma aminobutyric acid
GC-MS	Gas chromatography mass spectrometry
GHB	Gamma hydroxybutyric acid
GLUT4	Glucose transporter type 4
Gly	Glycine
GWA	Genome wide association
h	Hour
H&L	Heavy and light chain
H_2O	Water
H_2O_2	Hydrogen peroxide
HCl	Hydrogen chloride
HDL-C	High density lipoprotein cholesterol
HFHC	High-fat high-carbohydrate
HI	High intensity (80% VO_{2peak})
HIE	High intensity exercise
HIF-1α	Hypoxia inducible factor 1
HMDB	The human metabolome database
HOMA-IR	Homeostasis model assessment of insulin resistance
HRP	Horse radish peroxide
IgG	Immunoglobulin G
Ile	Isoleucine
IMTG	Intramuscular triglycerides
IRS1	Insulin receptor substrate 1

ISI	Insulin sensitivity index
ISTD	Internal standard
I.V.	Intravenous
IVGTT	Intravenous glucose tolerance test
kb	Kilobases
kcal	Kilocalories
KEGG	Kyoto encyclopedia of genes and genomes
kg	Kilograms
kgbw	Kilogram body weight
kJ	Kilojoules
K_m	Michaelis constant
LBM	Lean body mass
LC-MS	Liquid chromatography mass spectrometry
LDL-C	Low density lipoprotein cholesterol
LN_2	Liquid nitrogen
LO	Low intensity (40% VO_{2peak})
Lys	Lysine
M	Male
m/z	Mass-to-charge ratio
m^6A	N^6- methyladenosine
mA	Microamperes
MC4R	Melanocortin 4 receptor
MEF2A	Myocyte enhancer factor 2A
MeOH	Methanol
MeOX	Methyloxime
METTL3	Methyltransferase like 3
mins	Minutes
miRNA	Micro ribonucleic acid
ML-PLS-DA	Multilevel partial least squares discriminant analysis
MRI	Magnetic resonance imaging
mRNA	Messenger ribonucleic acid
MSI	Metabolomics standards initiative
mSv	Millisievert
mTORC1	Mammalian target of rapamycin complex 1
NaCl	Sodium chloride
NC	Negative control

NCBI	National centre of biotechnology information
NEFAs	Non-esterified fatty acids
NMC	Number of misclassifications
NMR	Nuclear magnetic resonance
O_2	Oxygen
OGL	Oral glucose load
OGTT	Oral glucose tolerance test
OLTT	Oral lipid tolerance test
PBQC	Pooled biological quality control
PDK4	Pyruvate dehydrogenase kinase, isozyme 4
PG	Plasma glucose
PGC1α	Peroxisome proliferator-activated receptor gamma coactivator 1-α
Phe	Phenylalanine
PKB	Protein kinase B
PL	Plasma lactate
PLS-DA	Partial least squares discriminant analysis
Pro	Proline
PVDF	Polyvinylidene fluoride
RER	Respiratory exchange ratio
RFU	Relative florescence units
RNA	Ribonucleic acid
ROC	Receiver operating characteristic
RP	Rank product
RPE	Rating of perceived exertion
RPM	Revolutions per minute
RQ	Respiratory quotient
RRR	Relative response ratio
RT-PCR	Real time polymerase chain reaction
s	Seconds
SAM	S-adenosylmethionine
SDS	Sodium dodecyl sulfate
SEM	Standard error of the mean
Ser	Serine
SNP	Single nucleotide polymorphism
T2DM	Type 2 diabetes mellitus
TBST	Tris-buffered saline with Tween 20

TCA	Tricarboxylic acid
TE	Tris ethylenediaminetetraacetic acid
TFAM	Mitochondrial transcription factor A
TG	Triglycerides
Thr	Threonine
T.I	Time interval
TMS	Trimethylsilyl
Tyr	Tyrosine
v.	Versus
Val	Valine
VIP	Variable importance for projection
VLDL	Very low density lipoprotein
VO_{2max}	Maximal oxygen consumption
VO_{2peak}	Peak oxygen consumption
W	Watts
x-FC	Fold change
YTHDF2	YTH N^6-methyladenosine RNA binding protein 2 (also known as the high-glucose-regulated protein 8)

CHAPTER ONE

Thesis Scope

There is ongoing debate as to whether genetic or environmental influences drive one of the modern world's biggest health issues, overweight and obesity, with the likelihood being that both factors contribute to the multifaceted problem. Twin studies have indicated polygenic factors to contribute towards up to 70% of the variation in obesity susceptibility between adults (Maes et al. 1997), and have shown genetic factors to determine adiposity outcomes in response to the environment (Hainer et al. 2000; Mustelin et al. 2009). Genome wide association (GWA) studies have provided clues as to which genes may have the greatest influences on predisposing some individuals to obesity. Since the initial GWA study to reveal a robust association between variants of the fat mass and obesity-associated (FTO) gene and obesity (Frayling et al. 2007), there has been a surge in reproducible association studies among varying populations (Jacobsson et al. 2012; León-Mimila et al. 2013; Mägi et al. 2013; Xi et al. 2013; Yako et al. 2015). To date, FTO harbours the strongest known susceptibility locus for obesity risk.

Knowledge of the cellular mechanisms by which FTO may function has increased rapidly in recent years due to technological advancements. However, evidence concerning the exact biological mechanisms linking FTO risk polymorphisms to obesity susceptibility remains unclear. Information regarding FTO's peripheral influences, including the effect of its function on skeletal muscle metabolism, is

limited. A known function of the FTO protein is its ability to catalyse the Fe^{2+}- and 2-oxoglutarate (2OG)- dependent nucleic acid demethylation of 3-methylthymine (3-meT) and 3-methyluridine (3-meU) in single stranded DNA and RNA, respectively (Gerken et al. 2007; Han et al. 2010). Furthermore, a more recently identified role of the FTO protein is its ability to demethylate N^6-methyladenosine (m^6A) (Jia et al. 2011), a developmentally and dynamically regulated post-transcriptional RNA modification (abundant in messenger RNA (mRNA)) that is reversible via the actions of methyltransferases and demethylases (see review by Niu et al. 2013). The post-transcriptional function of the FTO protein provides a mechanism for a combination of genetic and environmental influences on obesity susceptibility. Thus, investigations are required to explore how FTO's demethylating abilities may be integrated into the multifaceted network of energy metabolism control. Recent investigations have highlighted a role of FTO in nutrient sensing, with a downregulation of FTO resulting as a consequence of glucose and essential amino acid deprivation (Cheung et al. 2013), and linked to a decreased signalling of mammalian target of rapamycin complex 1 (mTORC1) (Gulati et al. 2013). Additionally, the activity of 2OG and the demethylase function of FTO, can be inhibited by high levels of tricarboxylic (TCA) cycle intermediates succinate and fumarate (Gerken et al. 2007; Xiao et al. 2012), suggesting a role of FTO in energy pathways. Furthermore, hepatic FTO protein expression levels have been indicated in the regulation of gluconeogenesis (Bravard et al. 2014; Guo et al. 2015; Poritsanos et al. 2010). The ubiquitous expression and apparent pleiotropic nature of FTO highlights the complexity of linking genetic polymorphisms to specific cellular and metabolic mechanisms to establish the causation behind genotype specific clinical associations.

Of interest are variants of the FTO rs9939609 polymorphism (T>A), where individuals homozygous for the risk A-allele have a ~1.67-fold increased risk of obesity (equating to ~3 kg excess body weight) compared to individuals homozygous for the non-risk T-allele (Frayling et al. 2007). However, the association between the risk A-allele and the odds of obesity has been found to be reduced by ~30% in physically active adults, compared to inactive adults (Kilpeläinen et al. 2011). The mechanisms underlying this finding may be related to a potential influence of exercise on FTO protein function and/or metabolic outcomes in skeletal muscle, and we speculate that this may differ between allelic variants of FTO. The influence of FTO on skeletal muscle metabolism is equivocal with studies suggesting both an association (Andreasen et al. 2008; Grunnet et al. 2009*b*; Wahl et al. 2014) and no association (Berentzen et al. 2008; Cecil et al. 2008; Grunnet et al. 2009*a*; Kjeldahl et al. 2014; Speakman et al. 2008; Zabena et al. 2009) between differing rs9939609 genotypes and metabolic outcomes. An age-dependent decline in skeletal muscle FTO mRNA expression has been linked to a decreased glucose oxidation and elevated fat oxidation in response to insulin infusion (Grunnet et al. 2009*b*). Interestingly, these peripheral characteristics are also observed in those with metabolic inflexibility, following insulin-stimulation (Kelley et al. 1999; Kelley et al. 1994; Kelley & Simoneau 1994). This may indicate a role of FTO in metabolic flexibility. Furthermore, skeletal muscle FTO mRNA expression has been positively associated with the transcription of nutritionally regulated metabolic genes in response to insulin-stimulation (Grunnet et al. 2009*b*). Thus, it could be speculated that FTO may too be a nutritionally regulated metabolic gene that controls metabolism by restoring metabolic functions upon environmental changes.

The investigation of metabolic interactions occurring in humans is complex and requires appropriate tools to detect and interpret the simultaneous interplay between genes and their ultimate effect on metabolism (Chorell et al. 2009). Modern technologies, such as metabolomics, can allow for a reduction in the need for large cohorts (which are typical of GWA studies) and may facilitate the observation of metabolic profiles associated with particular genes or genotypes. To date, published work utilising this approach has not identified differing metabolic signatures across allelic variants of FTO when analysing plasma samples taken whilst fasting (Kjeldahl et al. 2014; Wahl et al. 2014), with minimal genotypic differences observed in response to nutritional metabolic challenges (Wahl et al. 2014). No published study has induced acute perturbations to metabolic homeostasis using exercise to investigate whether differences in skeletal muscle metabolic profiles exist between allelic variants of FTO.

A major regulatory role of the FTO protein on energy metabolism and body weight regulation has been demonstrated in mouse models, with those encompassing a complete or partial loss of *Fto* function (Church et al. 2009; Fischer et al. 2009), as well as an overexpression of *Fto* (Church et al. 2010), exhibiting lean and obese phenotypes respectively. FTO overexpression in myotubes has been associated with similar metabolic defects to that observed in diabetic skeletal muscle, including enhanced lipogenesis, oxidative stress and a reduction in mitochondrial oxidative function (Bonnard et al. 2008; Bravard et al. 2011; Kelley & Simoneau 1994). Elevated skeletal muscle FTO mRNA and protein expression has been indicated for type 2 diabetes mellitus (T2DM) patients compared to obese and lean individuals (the latter of which showed lower and comparable expression levels) (Bravard et al. 2011).

These findings suggest that an elevated expression of FTO in skeletal muscle may not be related to the passive state of obesity per se, but rather to the muscle defects which characterise T2DM (Bravard et al. 2011). Whilst exercise is not a defect, it may exert a similar condition by driving alterations in glucose transport and metabolism. How FTO is regulated in response to exercise, as an acute metabolic stimulus, is unclear. It can be speculated that the ability to modulate FTO protein activity in response to metabolic stimuli may differ in those who are lean and obese, despite Bravard et al. (2011) finding these population subgroups to have similar levels of FTO protein expression. Furthermore, Bravard et al. (2011) did not classify participants based on FTO genotype, thus it is unknown whether differences in FTO protein expression or function existed between risk and non-risk variants of FTO. To date, research on human cohorts have only explored whether genotypic difference exist for skeletal muscle FTO mRNA expression (as opposed to FTO protein expression), with no differences evident in a basal state (Grunnet et al. 2009*a*; Grunnet et al. 2009*b*) or in response to insulin-stimulating conditions (Grunnet et al. 2009*b*). No published investigation has used exercise as an acute stimulus to observe whether differences across allelic variants exist for FTO mRNA and/or protein expression, or FTO protein function, in the skeletal muscle.

The studies presented in this dissertation were therefore designed to investigate the metabolic mechanisms of FTO, with a focus on perturbing energy flux using acute physiological stimuli (dietary and exercise stressors) to examine whether genotypic differences existed for metabolic flexibility, skeletal muscle metabolism, FTO mRNA and protein expression, and FTO function (measured as m^6A RNA methylation status).

CHAPTER TWO

Literature Review

2.1 The Obesity Epidemic

Overweight and obesity (defined as a body mass index (BMI) greater than 25 and 30 kg/m^2, respectively) are characterised by an abnormal or excessive accumulation of body fat (WHO 2000) and are becoming a major global public health burden. In 2013, the Australian Government published results from the National Health Survey showing that 70% of male and 56% of female adults were estimated as being either overweight or obese (ABS 2013). This has substantially increased from 63% and 47% for males and females, respectively, in 1995 (ABS 1997). Overweight and obesity are high risk factors for several chronic metabolic diseases including cardiovascular disease (CVD), hypertension, T2DM and some cancers, whilst also being associated with various biomechanical and psychosocial disorders (WHO 2000). The worldwide obesity epidemic accounts for a significant influence on the growing portion of health care spending in the industrial world. Data from the Australian Diabetes, Obesity and Lifestyle (AusDiab) study estimated the total annual cost of overweight and obesity to be $56.6 billion, with $21.0 billion contributing directly, and $35.6 billion via indirect costs (Colagiuri et al. 2010). As such, it is important to examine factors that influence the rapidly growing global prevalence of overweight and obesity.

The modern obesogenic lifestyle (in particular high fat and/or energy dense diets and low levels of physical activity) encompasses an environment that promotes body fat

accumulation. However, genetic variations can predispose some individuals to be more susceptible to developing obesity in a similar environment (Hainer et al. 2000; Maes et al. 1997; Mustelin et al. 2009). The heritability of this condition may explain circumstances where environmental exposure is similar between given populations, yet considerable disparity in individual BMI is observed (Ravussin et al. 1994; Rokholm et al. 2011). A meta-analysis of identical and non-identical twin studies was the first to attribute polygenic factors to up to 70% of the adult variation to obesity susceptibility (Maes et al. 1997). Subsequent identical twin studies have confirmed that genetic factors can determine changes in adiposity in response to environmental influences (Hainer et al. 2000; Mustelin et al. 2009), supporting the notion that obesity occurrence is likely to result through a complex interplay of both individual susceptibility and environmental/lifestyle factors. Although our understanding of this multifactorial condition is improving, the precise genetic–cellular relationships that form the basis of obesity risk still require much elucidation.

2.2 Experimental Methods for Genetic-Cellular Relationship Determination

2.2.1 Genome Wide Association (GWA) Studies

Humans carry the same base residue on both chromosomal homologs at the majority of genomic sites (approximately 99%) (LaFramboise 2009). The remaining variation in the genome encodes much of the diversity among humans (LaFramboise 2009), including individual differences in disease susceptibility and responses to changes in the environment, making each person unique. Whilst individual genetic factors have a small effect on obesity-susceptibility, the combined effects of allelic variations (both beneficial and disadvantageous) can exert a greater impact on an individual's predisposition to gain weight, particularly when coupled with environmental influences (Hess & Brüning 2014; Speliotes et al. 2010). As such, genetic factors require consideration in the understanding of obesity. Human genetic discovery methodologies, such as GWA studies, aim to improve our understanding of the variations in the human genome that influence the biologic mechanisms underlying polygenic diseases and traits. These variations can be characterised by numerous polymorphisms and mutations that alter genetic sequence and structure (i.e. single base exchanges, copy-number variations, deletions, inversions, insertions and duplications). Single nucleotide polymorphisms (SNP's) account for a major part of genetic variance by creating different combinations in DNA sequences (LaFramboise 2009). Analysis of SNP arrays can allow for the interrogation of a significant proportion of human genetic variation and has facilitated hundreds of GWA studies since the early 2000's. GWA studies typically involve the use of hypothesis free approaches to screen the whole genome, with the ultimate goal of identifying loci which implicate genetic variants that are statistically more prevalent in individuals

with a specific disease than in those free of the disease (i.e. linking a specific disease to a specific variation in DNA sequence) (LaFramboise 2009). A recent meta-analysis, which included association results for up to 339,224 individuals from 125 studies (including 82 GWA studies), identified 97 loci to account for 2.7% of variation in BMI (Locke et al. 2015). The fat mass and obesity associated (FTO) gene and melanocortin 4 receptor (MC4R) gene were found to have the strongest associations, with these loci key contributors to polygenic obesity (the more common clinical situation) and monogenic obesity (the rarer Mendelian form), respectively (Locke et al. 2015).

2.2.2 Recent Technological Advancements

Recent progress in the field of obesity genetics, in addition to technological advancements, provides the opportunity to explore novel and potentially unsuspected pathways related to this condition. Despite GWA studies being a powerful way of identifying genes associated with common diseases, it is often difficult to translate such findings into an understanding of how the gene products act at a cellular level. Many of the obesity loci identified by GWA studies do not harbour genes with clear connections to the biology of body weight regulation (Speliotes et al. 2010), and by only associating genetic variants with clinical outcomes little can be inferred regarding the underlying disease causing mechanisms. Furthermore, the effect size of genetic associations with clinical phenotypes is often small, and therefore large populations are needed to obtain sufficient statistical power for the identification and exploration of disease causing genetic variants (Kathiresan et al. 2008; Willer et al. 2008). The rapidly evolving field of metabolomics, in addition to functional genomic approaches (that investigate dynamic gene transcripts and protein functions), may

contribute to solving these issues by elucidating trends in the end products of genetic variation (Figure 2.1).

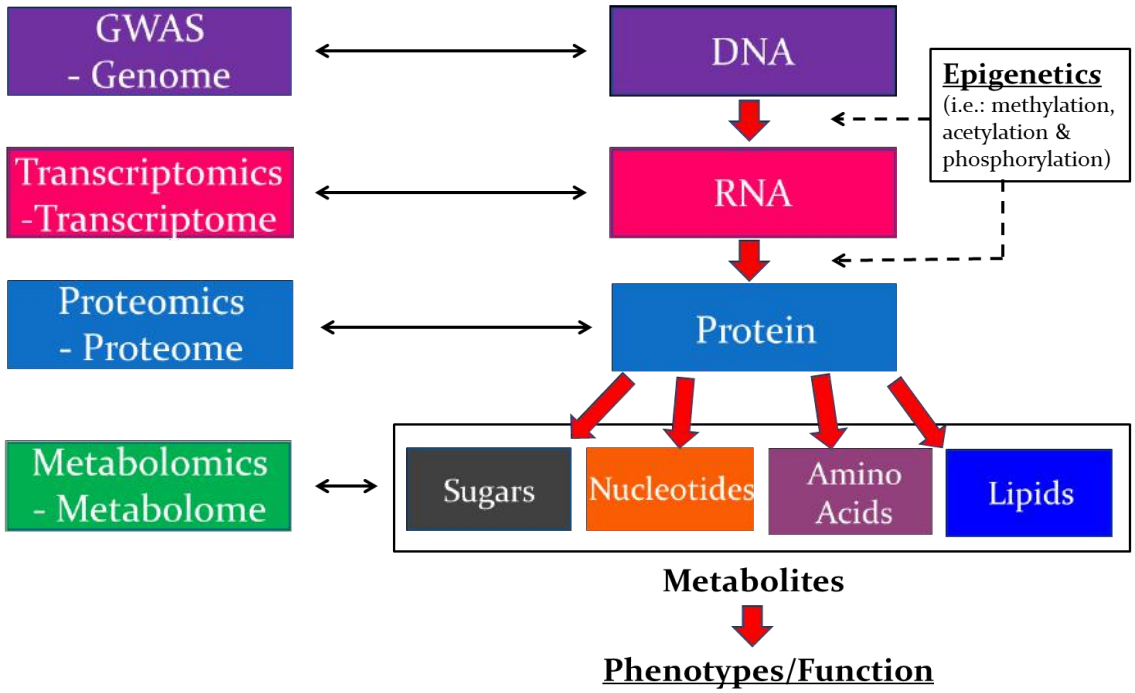


Figure 2.1 The formation of metabolites are influenced by DNA.

Metabolomics refers to "the quantitative measurement of the dynamic multi-parametric metabolic response of living systems to pathophysiological stimuli or genetic modification" (Nicholson et al. 1999). This involves characterising and quantifying a large number of metabolites and other small molecules that are present in a biological sample at one time (Nicholson et al. 1999). Genetic variants that associate with changes in key lipids, carbohydrates or amino acids are expected to display much larger effect sizes due to their direct involvement in metabolite conversion modification (Gieger et al. 2008). Additionally, this approach can be expected to provide insight into potentially affected candidate pathways and generate a metabolic signature reflecting an individual state and/or disease (Gieger et al. 2008;

Park et al. 2015). By utilising an untargeted metabolomics approach, the characterisation of core metabolic changes commonly covered in targeted approaches can be achieved in addition to detecting previously unknown or poorly characterised metabolites (Boughton et al. 2011). The use of this modern technology may therefore assist in elucidating the underlying molecular disease-causing mechanisms by which known risk loci, such as FTO, are associated with the development of obesity.

2.3 The Fat Mass and Obesity-Associated (FTO) Gene

Since the initial GWA studies to fuel interest in the FTO gene and obesity predisposition were published in 2007 (Dina et al. 2007; Fraying et al. 2007), numerous investigations have focused their attention on determining the potential mechanism(s) by which FTO influences obesity susceptibility. The story of FTO illustrates how integrating lessons learnt through GWA studies with functional genomic and metabolomics technologies can markedly increase our understanding of the physiology of obesity. Although some mechanistic roles of FTO have been discovered within the past 9 years, including its demethylation and nutrient sensing abilities, there is still a lot we don't know about how the known functions of FTO may contribute to obesity susceptibility, and whether there are additional unknown mechanisms at play.

2.3.1 FTO Properties

FTO was first identified in 1999 as one of the six genes deleted in fused toe mice (Peter et al. 1999). Ironically, this gene was initially referred to as 'Fatso' on account of its large size prior to any indication of a link to obesity (Fischer et al. 2009; Peter et al. 1999). The human FTO gene encompasses nine exons, which span more than 400 kilobases (kb) on chromosome 16 (region 16q12.2) (Figure 2.2) (Stratigopoulos et al. 2008). The detected obesity risk variants of FTO are located within a 47 kilobase (kb) region, which encompasses the first two introns as well as the second exon of the FTO gene (Dina et al. 2007; Fraying et al. 2007; Stratigopoulos et al. 2008). The most commonly studied obesity risk polymorphism, rs9939609, is in the first intron of the FTO gene. The predicted human FTO protein consists of 505 amino acids and is localised to the cell nucleus (Han et al. 2010). The crystal structure of the FTO protein comprises two domains: 1) a N-terminal domain that contains a catalytic core, and 2) a C-terminal domain that is thought to play a role in stabilising the N-terminals conformation and catalytic ability (Han et al. 2010). A fully functional FTO protein is critical in the developmental stage, as an impaired enzymatic function of FTO can cause postnatal growth retardation, microcephaly and brain malformations, functional brain deficits, severe psychomotor delay, facial malformations, cardiac deficits and premature death (Boissel et al. 2009). Furthermore, the FTO protein has been shown to belong to the DNA repair enzyme alkylatin B (AlkB) family, and can catalyse the Fe^{2+}- and 2OG-dependent demethylation of 3-methylthymine (3-meT) and 3-methyluridine (3-meU) on single stranded DNA and RNA respectively (Gerken et al. 2007; Han et al. 2010). The FTO protein can also demethylate N^6-methyladenosine (m^6A) (Jia et al. 2011); a widespread post-transcriptional modification largely found on messenger RNA (mRNA). It is the structural basis of the FTO protein (i.e. the N-

and C-terminal domains, an extra loop covering one side of the conserved jelly-roll motif, and the ability to form hydrogen-bonding interactions with carbonyl oxygen atoms in 3-meT and 3-meU) that is thought to influence FTO's specificity for single stranded nucleic acids, in particularly RNA, as its preferred substrate (Han et al. 2010). Furthermore, the post-transcriptional role of FTO gives weight to the potential for environmental influences to play a part in influencing genetic susceptibility to obesity.

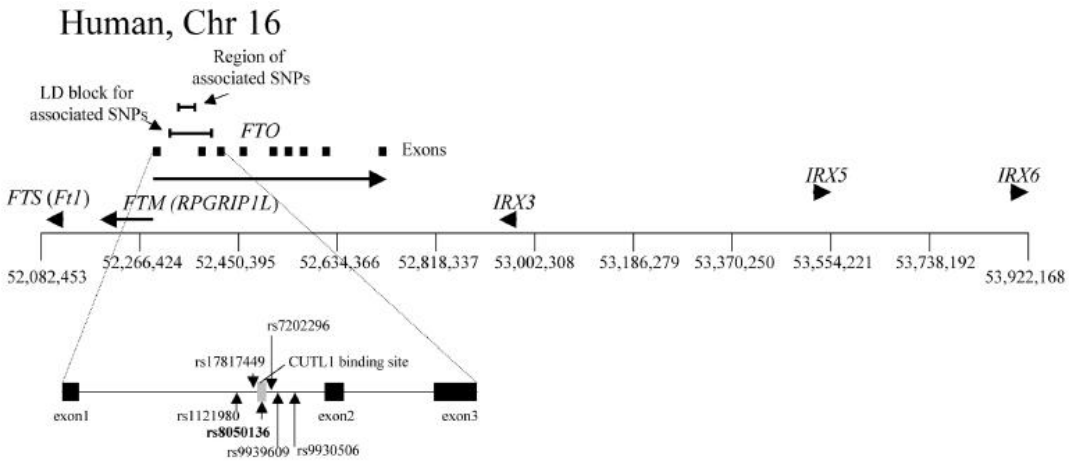


Figure 2.2 Position of FTO in the human genome, with neighbouring IRXB cluster genes and the FTM (RPGRIP1L) gene. FTO SNPs associated with BMI are indicated, including rs9939609. Reproduced from Stratigopoulos et al. 2008.

There is also evidence to suggest that associations with obesity may be mediated through complex and long-range regulatory effects between FTO and alternate genes within close proximity to the FTO locus. Of interest is RPGRIP1L, which is located approximately 3.4 kb upstream of FTO in humans, and IRX3 and IRX5, which are located approximately 500 kb and 1.2 Mbp downstream of FTO, respectively (Figure 2.2) (Stratigopoulos et al. 2008). FTO variants linked with obesity have been associated with increased expression of IRX3 and IRX5 in adipocytes, and a consequential reduction in energy expenditure via thermogenesis and increased lipid

storage (Claussnitzer et al. 2015). This suggests an interaction between FTO, IRX3, and IRX5, which, when manipulated, may influence adipocyte regulation and exert pro-obesity and anti-obesity effects (Claussnitzer et al. 2015). It has also been hypothesised that human obesity may be influenced by FTO SNPs via an effect on RPGRIP1L expression and ciliary function. RPGRIP1L is located in the basal body of cilia (Vierkotton et al. 2007), and ciliary defects have been associated with obesity in mice and humans (Davenport et al. 2007). Additionally, the disruption of genes involved in cilia formation and maintenance have been associated with the development of obesity (Davenport et al. 2007). However, no difference in adiposity has been reported in mice homozygous for a targeted mutation in Rpgrip1l (Peters et al. 1999; Vierkotton et al. 2007), or with mutations in human RPGRIP1L compared to the general population (Arts et al. 2007). Furthermore, *Fto* deficient mice, which exhibit reduced body weight and fat mass, have unaltered mRNA expression levels of Rpgrip1l mRNA (Fischer et al. 2009), thus associations with adiposity are likely driven by a direct effect of FTO. Whilst this thesis does not explore whether FTO polymorphisms are associated with obesity through a direct regulatory effect on these genes or an indirect effect (e.g. through a regulatory site variation) on their function, it is important to highlight the complexity of the genomic context when investigating trait-associated genetic variants.

2.4 FTO and Obesity Susceptibility

2.4.1 FTO Identification Using GWA Studies

Frayling and colleagues (2007) were the first to identify several SNP's of the FTO gene to be associated with adiposity in humans using a GWA study. The initial identification of FTO as a risk candidate gene was due to an association with the prevalence of T2DM (Frayling et al. 2007). However, after adjusting for BMI this association was removed and it was concluded that any link between FTO and T2DM was an indirect result of FTO influencing BMI (Frayling et al. 2007). An association between common variants in the first intron of the FTO gene and obesity, as well as obesity related phenotypes (including increased leptin levels, decreased high density lipoprotein cholesterol (HDL-C), subcutaneous fatness, total fat mass, and hip and waist circumference) has since been replicated in numerous human cohorts (Andreasen et al. 2008; Kring et al. 2008; McCaffery et al. 2012; Tanofsky-Kraff et al. 2009). Additionally, SNP's in the first intron of the FTO gene have been correlated with a higher BMI in both children and adults, regardless of gender, and have been confirmed in multiple ethnicities (Jacobsson et al. 2012; León-Mimila et al. 2013; Mägi et al. 2013; Xi et al. 2013; Yako et al. 2015).

2.4.1.1 FTO rs9939609 polymorphism

Of interest are variants of the FTO rs9939609 polymorphism (T>A), which lies within the first intron and has a minor allele frequency of 0.45 (Frayling et al. 2007). Consistent with Hardy–Weinberg expectations, 17% of individuals are homozygous and 49% heterozygous for the risk A-allele of this polymorphism (Tanofsky-Kraff et al. 2009). Individuals homozygous for the rs9939609 risk A-allele were found to have a ~1.67-fold increased risk of obesity, equating to ~3 kg excess body weight, compared with those who had not inherited the risk A-allele (TT genotypes) (Frayling et al. 2007). The effect of this polymorphism on BMI has been observed from childhood (approximately 7 years of age) to old age (approximately 74 years of age) (Frayling et al. 2007). Life course studies have found the association between the FTO rs9939609 polymorphism and BMI to strengthen throughout adolescence, and peak in the early 20's (Hardy et al. 2010), with this association weakening with age (Hardy et al. 2010; Jacobsson et al. 2011; Qi et al. 2008). Interestingly, whilst the expected association between the FTO rs9939609 polymorphism and BMI in childhood was observed in a meta-analysis by Sovio et al. (2011), this was only following an inverse association between the same polymorphism and BMI in infancy (those younger than 2.5 years). Those with the risk A-allele were found to have an earlier adiposity rebound (the rapid rise in BMI that typically occurs between the age of 5 and 7 years) compared to TT genotypes (Sovio et al. 2011), which is associated with an increased risk of metabolic diseases such as obesity and T2DM in adulthood (Rolland-Cachera et al. 2006).

2.4.2 FTO's Influence on Eating Behaviour

FTO is ubiquitously expressed in human tissues, with high levels apparent within the hypothalamic site of the brain that controls food intake and regulates energy balance (Morton et al. 2006). Research to date has largely focused on the potential for FTO to influence obesity predisposition via central nervous system mechanisms (i.e. by altering energy balance) (McCaffery et al. 2012; Tanofsky-Kraff et al. 2009; Wardle et al. 2008). Multiple studies have proposed that the hypothalamic expression of FTO may influence food intake control (including appetite regulation and eating behaviours), as well as whole body energy metabolism and homeostasis (Berentzen et al. 2008; Church et al. 2009; Haupt et al. 2008; McCaffery et al. 2012; Tanofsky-Kraff et al. 2009). Additionally, the FTO rs9939609 polymorphism has been indicated in the modulation of neural responses to food images in the brains of humans (who have similar adiposity and a lean BMI (<25 kg/m^2)), as indicated by blood oxygen level dependent (BOLD) observations via functional magnetic resonance imaging (MRI) scans (Karra et al. 2013). These findings showed a striking divergence in neural responsivity to food cues within brain regions linked to reward and behavioural control in participants homozygous for the risk A-allele compared to those with the TT genotype (particularly in the hypothalamus where high levels of FTO are found), which in turn, may underlie food preferences and obesity predisposition (Karra et al. 2013).

Several studies in paediatric, adolescent and adult cohorts have found FTO rs9939609 risk A-allele carriers to consume higher levels of total energy than those homozygous for the T-allele (Speakman et al. 2008; Tanofsky-Kraff et al. 2009; Timpson et al. 2008; Wardle et al. 2008; Wardle et al. 2009). A paediatric meta-analysis determined

the effect per A-allele to be a 14.3 kcal (59.8 kJ) increase in total energy intake daily (Qi et al. 2015). Whilst this heightened daily energy intake is not likely to directly lead to continuous weight gain, regularly exceeding individual energy requirements in small amounts may offset energy balance, leading to additional intake and eventual weight gain (Hill et al. 2009). Additionally, if impairments to metabolic flexibility were a mechanism by which FTO contributes to obesity susceptibility, an inability to effectively utilise energy provided from dietary sources (in response to its availability) may lead to increased adiposity overtime. Moreover, there is evidence to suggest that risk A-allele carriers have a greater preference for, and consumption of, energy dense foods (Brunkwall et al. 2013; Cecil et al. 2008; Sonestedt et al. 2009; Tanofsky-Kraff et al. 2009; Timpson et al. 2008; Wardle et al. 2009), engage in prolonged snacking post meal consumption (Wardle et al. 2009), and have a reduced satiety rating (Wardle et al. 2008) compared to TT genotypes. However, these findings are inconsistent with others who have not found differences between FTO rs9939609 genotypes and total energy intake, or the percentage of intake from energy dense foods, in children, adolescents or adults (Bauer et al. 2009; Hakanen et al. 2009; Liu et al. 2010). Food intake and preference is complex, with many determinants influencing eating behaviours on both a household and population based level (Thomas 1991). As such, the ability to confirm or disprove any association between FTO and eating behaviour should involve an integrative analysis approach that combines other biological, environmental, political and social determinants of health. Furthermore, several peripheral pathways have been proposed as important feedback systems that can affect overall eating behaviour, such as peripheral glucose utilisation influencing satiety signalling (see review by Stubbs 1999). Nutrient metabolism should therefore not be overlooked when investigating the mechanisms of FTO.

2.5 Metabolism

Metabolism is defined as the sum of chemical processes occurring within a living organism. This includes the generation of energy from the breakdown of substrates (carbohydrate, fat and protein) into a usable form. The preferred metabolic pathway chosen to produce energy at any given time is dependent on substrate availability in addition to the acute availability of oxygen in the cell (hence aerobic or anaerobic production).

2.5.1 Skeletal Muscle Metabolic Flexibility

Skeletal muscle is a dynamic metabolic tissue with a high-energy turnover. In a healthy lean individual, skeletal muscle tissue accounts for approximately ~55% of individual body mass and plays a fundamental role in whole body energy metabolism and substrate turnover (see review by Zierath & Hawley 2004). Under normal physiological conditions the skeletal muscle relies on a blend of both fat and carbohydrate substrates as fuels for oxidative metabolism, with the proportional mix of fuels utilised influenced by multiple factors, including nutrient availability, nutrient induced hormone secretion, and contractile status (Zierath & Hawley 2004).

An increased acute availability of carbohydrate results in a concurrent increase in its oxidation, with a concomitant suppression of fat oxidation (Horton et al. 1995). The ability to control energy balance, and hence refrain from developing obesity due to long term positive shifts in this ratio, is highly dependent on ensuring that the fuel mixture being oxidised is equal to the fuel mixture being consumed (Flatt 1988; Hall et al. 2012). The ability of the skeletal muscle to move between the predominant

oxidation of fat in a fasted state, to the use of carbohydrates in a prandial state driven by insulin stimulation, is considered 'metabolic flexibility' (Aucouturier et al. 2001; Storlien et al. 2004). A slower switch between predominately oxidised substrates in a fasted to an insulin-stimulated state has been related to the pathogenesis of insulin resistance in skeletal muscle (Figure 2.3) (Kelley 2005; Kelley et al. 1999). An elevated respiratory quotient (RQ) measurement whilst fasting (reflecting elevated carbohydrate oxidation at a cellular level) is typically observed in individuals with metabolic inflexibility (Kelley et al. 1999). Respiratory exchange ratio (RER) measurements via gas exchange analysis can serve a non-invasive equivalent of measuring RQ at rest (Albouaini et al. 2007). Metabolic inflexibility also typically exhibits reduced insulin stimulated skeletal muscle glucose uptake and an inability to move between fuel sources appropriately when challenged by dietary stressors (Kelley et al. 1999). A diminished capacity to adjust fuel oxidation based on availability may translate into a greater positive energy balance, compounding weight gain over time (Zurlo et al. 1990), and consequently increasing the risk of developing obesity and its associated comorbidities. Additionally, a prolonged excessive demand on fat storing adipocytes can result in a "spill over" effect into non-adipose cells, including skeletal muscle cells which lack necessary storage space and can therefore suffer chronic lipotoxicity and consequential cellular dysfunction (Mittendorfer 2011).

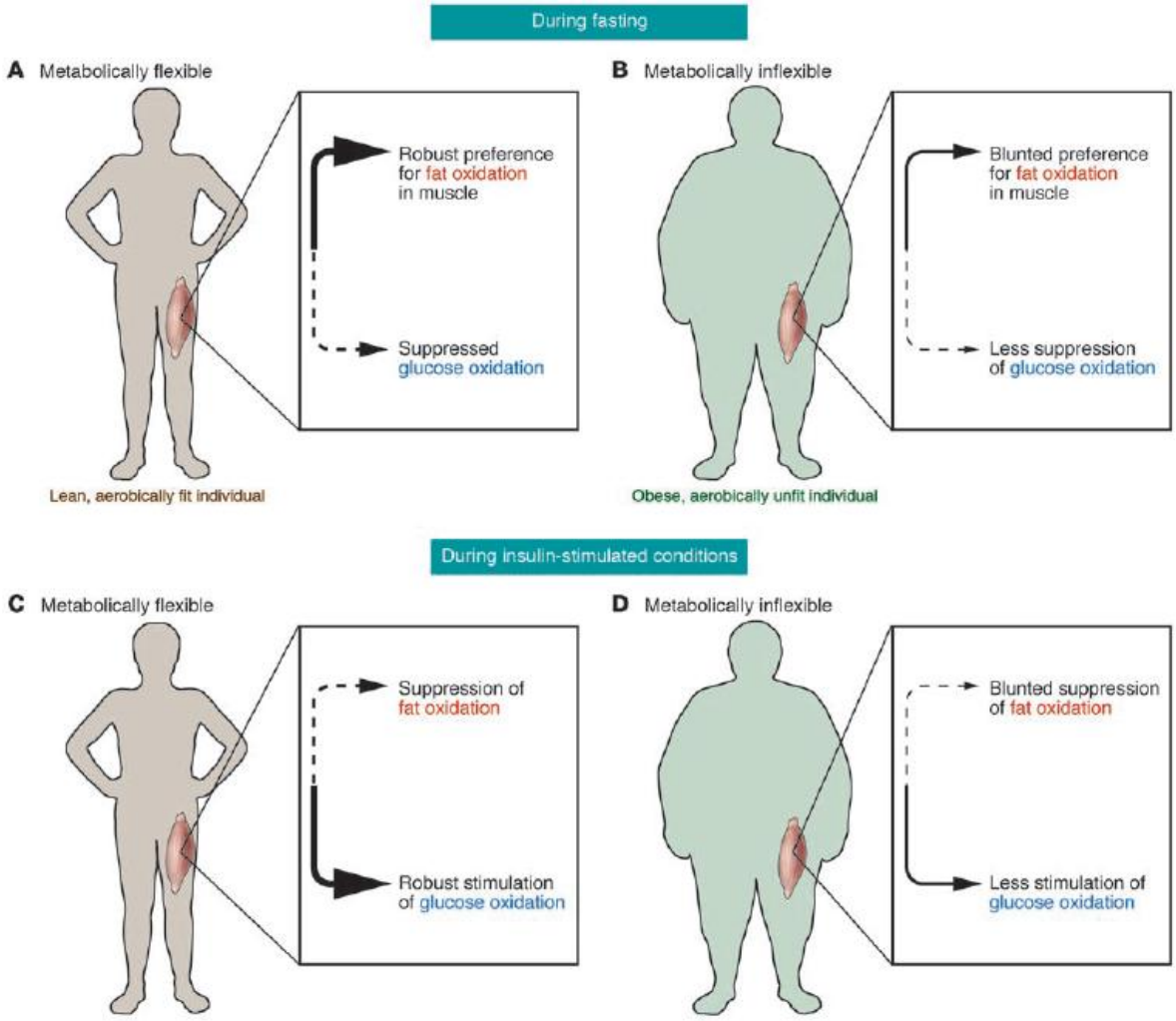


Figure 2.3 The flexibility of the skeletal muscle to switch between oxidising fat and glucose based on substrate availability is impaired in obese, aerobically unfit individuals. Reproduced from Kelley 2005.

During fasting "… in a lean, aerobically fit individual (**A**), in whom there is a high reliance upon fat oxidation during fasting conditions… in an obese, sedentary individual (**B**), in whom there is less reliance on fat and a greater reliance on glucose oxidation" (Kelley 2005). During insulin stimulated conditions "In skeletal muscle of a lean, aerobically fit individual (**C**), insulin strongly suppresses fat oxidation and induces a high reliance upon glucose oxidation… in skeletal muscle of an obese, sedentary individual (**D**), there is less stimulation of glucose oxidation by insulin and blunted suppression of fat oxidation" (Kelley 2005).

The concept of metabolic inflexibility may also extend to metabolism during exercise. The skeletal muscle is characterised by a high degree of plasticity in its ability to adaptively respond to environmental stressors that challenge the metabolic demands of the tissue (Barrès et al. 2012). Exercise results in a dynamic alteration in the demand for fuel and consequently stimulates a rapid change in the constitution of substrate supply being oxidised. The typical response in a basal state is to shift from utilising fat to carbohydrate during the transition from rest to exercise of increasing intensity, as fat cannot be oxidised at high enough rates to be the sole supplier of energy during moderate to vigorous exercise (van Loon et al. 2001). A shift from fat to carbohydrate oxidation can be represented by an elevated RER. Over time during a constant exercise workload there is also a potential to drift back towards utilising a greater ratio of fat for fuel, and thus a decrease in RER, to accommodate for the increased energy expenditure required (Bier & Young 1983; Romijn et al. 1993). A decreased ability to shift between fuel sources when transitioning from rest to submaximal exercise occurs in obese individuals with impaired glucose tolerance, compared to those with normal glucose tolerance (Prior et al. 2014). Additionally, the ability to alter substrate oxidation in response to environmental stimuli has been proposed to depend on genetically determined factors that may influence the balance between cellular oxidation and substrate storage capacities (Galgani et al. 2008*b*).

2.6 FTO and Metabolism

A complete loss of FTO protein function in humans is rare and associated with premature death (prior to 3 years of age) following growth abnormalities (Boissel et al. 2009), demonstrating a critical role of FTO for survival. Complete knockout of the *Fto* protein in mice further highlights its vital importance, resulting in a non-viable and severely compromised organism, which exhibits premature postnatal death, postnatal growth retardations, reductions in adipose tissue and lean mass, and increases in energy expenditure and sympathetic nervous system activity (Fischer et al. 2009). Similar findings are also observed when deleting *Fto* in the central nervous system (Gao et al. 2010), highlighting an apparent pleiotropic nature of the FTO protein, including a fundamental role in energy metabolism and body weight regulation. Although animal models have proven invaluable in the study of FTO thus far, the significant morbidity and early mortality caused by deleterious mutations makes it naive to assume that these models alone can provide us with a complete picture of FTO's mechanisms. Whilst no model allows for a direct comparison to humans (whom typically do not have a complete loss of FTO function), partial loss of *Fto* function in mice (via a missense point mutation) results in a milder phenotype of reduced fat mass without complications observed in deleterious/complete knockout models (Church et al. 2009). Due to the limitations of deleterious models, mechanistic data requires further validation through human studies (or at least through partial loss of *Fto* function models), before conclusions are drawn on FTO's potential effect on metabolic pathways. Currently, the influence of allelic variations of the FTO rs9939609 polymorphism on metabolism is equivocal, with previous human investigations suggesting both an association (Andreasen et al. 2008; Grunnet et al. 2009*b*; Wahl et al. 2014) and no association (Berentzen et al. 2008; Cecil et al. 2008;

Grunnet et al. 2009*a*; Kjeldahl et al. 2014; Speakman et al. 2008; Zabena et al. 2009) between differing genotypes and skeletal muscle metabolism. Findings from human and animal investigations that explore the potential metabolic functions of FTO are described in the following section.

2.6.1 FTO's Influence on Energy Expenditure and Substrate Metabolism

The reported influence of FTO on energy expenditure is inconsistent between human and animal investigations (Berentzen et al. 2008; Cecil et al. 2008; Church et al. 2009; Corpeleijn et al. 2010; Fischer et al. 2009; Grunnet et al. 2009*a*; Speakman et al. 2008). To date, no human study has reported an association between allelic variants of the FTO rs9939609 polymorphism and energy expenditure in either a basal (Berentzen et al. 2008; Grunnet et al. 2009*a*; Speakman et al. 2008) or postprandial state (Berentzen et al. 2008; Cecil et al. 2008). Additionally, investigations on human cohorts have found no evident relationship between the FTO rs9939609 polymorphism and the ability to regulate substrate oxidation in a basal state or in response to nutritional stimuli such as a high fat load and a euglycemic hyperinsulinemic clamp (EHIC) (Corpeleijn et al. 2010; Grunnet et al. 2009*b*). Grunnet et al. (2009*a*) compared homozygous carriers of the risk A-allele to the combined data sets of AT and TT genotypes when reporting that no genotype specific association existed for energy expenditure, or glucose or fat oxidation, when observing participants over a 24-hour period. At this point it is unclear whether grouping heterozygotes with controls would have an implication on results, as the risk A-allele has been associated with increased odds of being obese, and with obesity related traits (including increased waist circumference and fat mass), even in its heterozygous form (Andreasen et al. 2008; Frayling et al. 2007; Tanofsky-Kraff et al.

2009). Furthermore, Corpeleijn et al. (2010) did not find an association between the FTO rs9939609 polymorphism and the ability to regulate fat oxidation in a basal state or during a high fat load dietary challenge. These findings may be influenced by Corpeleijn and colleagues (2010) only observing individuals who were considered obese (with no lean control group), as a reduced capacity for obese individuals to oxidise fat has previously been detected and linked to a greater insulin resistance in these individuals compared to lean (Kelley et al. 1999). By examining a wider BMI range, selecting suitable control groups, and using analysis of covariance (ANCOVA) when performing statistics, it may be possible to clarify whether allelic variants of FTO influence skeletal muscle fat and/or glucose oxidation. It is also important to consider whether gender could affect study outcomes, as the influence of FTO variants (other than rs9939609) on BMI has been found to be restricted to males and postmenopausal females in a Slavic population (Hubacek et al. 2009), and to middle aged women in a Mexican population (Saldaña-Alvarez et al. 2016). A gender specific regulation of the FTO rs9939609 polymorphism has been indicated in a Dutch adult cohort, with each A-allele found to be associated with a 1.99× higher risk of men gaining weight over a 10-year period, compared to women (Bouwman et al. 2014). Furthermore, when observing the effects of FTO rs9939609 variants in a paediatric cohort, a gender specific association was found with obesity and BMI among young girls, but not young boys (Jacobsson et al. 2008). Jacobsson et al. (2008) also detected gender differences in the association of the FTO rs9939609 polymorphism with obesity related traits such as insulin sensitivity and plasma glucose, suggesting that FTO may have a gender specific influence in the development of insulin resistance, as well as obesity, in children. Thus, gender may require consideration when interpreting results. In addition, it is well known that a

limitation to indirect calorimetry is that multiple factors may influence the reliability of results, such as the consumption of food and nutrient containing beverages, smoking, some medications, and engaging in physical activity for varying times before measurement (Compher et al. 2006). As such, participant deviations from specific pre-measurement conditions set by researchers may influence scientific variability when using indirect calorimetry to investigate energy expenditure and substrate oxidation.

Whilst human studies to date have not detected an association between FTO genotypes and energy expenditure or substrate oxidation, investigations using genetically engineered mouse models, in which the function and/or levels of the *Fto* protein has been either eliminated (Fischer et al. 2009; Gao et al. 2010; Wu et al. 2010), reduced (Church et al. 2009), or enhanced (Church et al. 2010), have provided support for a role of *Fto* on metabolic processes. Fischer et al. (2009) observed a targeted knockout of the *Fto* protein in mice, which caused a loss of demethylase function, to demonstrate a direct role of *Fto* in energy homeostasis. The absence of the *Fto* protein resulted in reduced food intake, elevated energy expenditure, a loss of lean mass, and a reduced RER (likely representing protein catabolism) (Fischer et al. 2009). Similarly, a global partial loss of *Fto* function (via a dominant point mutation in the C-terminal domain, 1367F) has been shown to increase daily energy expenditure in mice, when compared to control littermates (Church et al. 2009). On the other hand, hypothalamic knockout of *Fto* does not alter body composition, energy expenditure or RER yet decreases food intake (McMurray et al. 2013). Taken together, these findings indicate that a reduction or removal of *Fto* in the hypothalamus may explain only a small part of the phenotype observed in the global

Fto knockout and partial loss of function models, indicating that *Fto* also promotes its biological effects through alternative, non-hypothalamic pathways.

Although the mouse models of complete and partial loss of *Fto* function used by Fischer et al. (2009) and Church et al. (2009) respectively resulted in increased energy expenditure, McMurray et al. (2013) found energy expenditure to be unchanged in adult onset global *Fto* knockout models. This finding followed regression adjustment using ANCOVA to account for observations of decreased lean body mass (McMurray et al. 2013). The difference in results reported by the researchers may be due to variations in the methodologies used to interpret energy expenditure data, particularly in situations where alterations to body composition changes have occurred, and has since been the subject of much debate (Kaiyala & Schwartz 2011; Müller et al. 2013). Whilst multiple linear regression of body composition parameters using ANCOVA is recommended for use in mouse models (as an energy expenditure normalisation method) (Tschöp et al. 2011), earlier data indicating *Fto* knockout to increase energy expenditure by Fischer et al. (2009) used an analysis of variance (ANOVA) for normalisation (which does not account for changes in lean body mass over time). Despite this, the use of the ANCOVA method for the normalisation of lean body mass did support that a specific deletion of *Fto* in mice can lead to an increased metabolic rate when adopted by Gao et al. (2010).

Mouse models with a partial loss of *Fto* function (Fto^{I367F} models) have also been found to have altered substrate metabolism, as indicated via an increase in RER, reflecting a switch to a relatively greater contribution of carbohydrate metabolism at 18 weeks of age (Church et al. 2009). Additionally, a partial loss of *Fto* function

results in a phenotype of reduced body weight and fat mass, with no difference in lean mass observed between mutant and wild-type mice (Church et al. 2009). Compared to the reported loss of lean mass observed in mouse models with complete *Fto* knockout (Fischer et al. 2009; McMurray et al. 2013), the partial loss of *Fto* function models used by Church and colleagues (2009) may more accurately reflect the effect of FTO variants in humans. Furthermore, the lean phenotype observed with a partial loss of *Fto* occurred despite no evident changes to physical activity or food intake (Church et al. 2009). Thus, *Fto* may regulate body weight status potentially due to an influence on whole body metabolism (Church et al. 2009), for example, by elevating body temperature or heat shock protein activity. It is also possible that elevations of energy expenditure and RER observed with a partial loss of *Fto* function may occur secondary to changes in substrate supply to the muscle (Church et al. 2009). This speculation can be made due to an elevation of fatty acid synthase (FASN) mRNA expression also being observed in the skeletal muscle of partial loss of *Fto* function mouse models, suggesting that fat synthesis may be enhanced in this tissue (Church et al. 2009). However, no increase in fat storage has been detected in partial loss of *Fto* function mouse models, and as such Church et al. (2009) speculated that elevations of RER, and a resultant lean phenotype, could alternatively occur secondary to a decreased supply of fatty acids to the muscle. Conversely, a significantly lower RER has been observed in germline and adult onset *Fto* knockout mice, with the metabolic fuel switch reflecting an increased protein and/or fat utilisation at the expense of carbohydrates (McMurray et al. 2013). The reduced RER observed in these *Fto* knockout models is most probably a reflection of delayed lean muscle growth (McMurray et al. 2013). Although the findings by McMurray et al. (2013) and Church et al. (2009) are conflicting, likely due to the differing effects of a partial and

complete loss of *Fto* on lean body mass, these studies suggest an influence of FTO on the regulation of peripheral metabolism and substrate utilisation.

In contrast to the increase in energy expenditure typically observed in complete *Fto* knockout (Fischer et al. 2009; Gao et al. 2010) and partial loss of *Fto* function models (Church et al. 2009), mice overexpressing *Fto* become obese and hyperphagic, whilst exhibiting unaltered energy expenditure compared to control littermates (once corrected for body fat and lean mass via ANCOVA) (Church et al. 2010). This finding may shed light on observations from human investigations (in which FTO protein expression or function is not measured) that have found obesity predisposing risk variants of FTO to be associated with an increased food intake, but to not appear to influence energy expenditure (Cecil et al. 2008; Speakman et al. 2008).

The measurement of energy expenditure and substrate utilisation in mouse model investigations has provided support for a role of FTO in energy metabolism. However, data obtained indirectly from the analysis of respiratory gas exchange alone cannot identify the specific cause(s) of metabolic dysfunction when impairments to energy expenditure and/or substrate oxidation are indicated. Research that uses biological samples to investigate FTO's influence on specific metabolic pathways may help solve this problem.

2.6.2 FTO's Influence on Metabolic Outcomes via Biological Sample Analysis

The measurement of metabolites in biological samples such as plasma and muscle may assist in determining the potential metabolic mechanisms linking FTO to obesity susceptibility. Blood and plasma glucose concentrations have been found to be similar between FTO rs9939609 genotypes when fasting (Speakman et al. 2008; Zabena et al. 2009), and in response to a glucose-stimulated oral glucose tolerance test (OGTT) (Andreasen et al. 2008; Berentzen et al. 2008). However, conflicting data in healthy adult males has shown an association between elevated fasting blood glucose concentrations and the FTO rs9939609 risk A-allele in its homozygous form, compared to variants encompassing the non-risk T-allele (Grunnet et al. 2009*a*). Grunnet et al. (2009*a*) also showed the AA genotype to be associated with hepatic insulin resistance in the aforementioned cohort, whilst Zabena et al. (2009) reported a similar level of insulin resistance between FTO genotypes. The findings by Grunnet et al. (2009*a*) are comparable to that of others who observed the AA genotype to be associated with decreased insulin sensitivity, in comparison to the TT genotype (Andreasen et al. 2008; Kring et al. 2008). Additionally, Andreasen and colleagues (2008) found the impact of FTO rs9939609 genotype on BMI to be highly influenced by insulin sensitivity, with a low insulin sensitivity index (ISI) enhancing the effect of genotype, particularly among homozygous risk A-allele carriers. Although there are numerous mechanisms that can lead to decreased insulin sensitivity, these results suggest that the ability of the skeletal muscle to uptake glucose in conditions driven by insulin may differ between allelic variants of FTO (Andreasen et al 2008).

2.6.2.1 Metabolic Signatures of FTO

Metabolomic techniques can be used as an untargeted approach to concurrently determine multiple metabolites present in biological samples. These include gas chromatography mass spectrometry (GC-MS), liquid chromatography mass spectrometry (LC-MS), and nuclear magnetic resonance (NMR) technology. Studies using metabolomics to investigate FTO genotype specific metabolic correlations began to emerge in 2014, and to date these investigations have had limited success in identifying associations between FTO polymorphisms and allelic specific metabolic signatures (Kjeldahl et al. 2014; Wahl et al. 2014).

Kjeldahl et al. (2014) found similar metabolic profiles between FTO rs9939609 genotypes when using NMR to examine plasma samples collected from 1000 Danish twins. The authors concluded that they had not been able to identify reliable markers of FTO genotypes due to intra-individual metabolic differences resulting from a "very complex network of interactions between genetic and environmental factors", indicating that at a systems level there is a "long path from genotype to metabolic profile" (Kjeldahl et al. 2014). However, as only fasting samples were analysed, potential metabolic signature differences between FTO genotypes may have been concealed behind tight homeostatic regulation (van Ommen et al. 2009).

Similarly, LC-MS testing of plasma samples collected from 56 healthy male adults did not show a unique metabolic profile of the FTO rs9939609 polymorphism following an overnight fast (Wahl et al. 2014). To allow for a detailed characterisation of FTO's influence on postprandial metabolism Wahl et al. (2014) also investigated whether metabolic profile differences existed between allelic homologues of FTO in

response to diverse intravenous and nutritional challenges (a OGTT, a standardised high-fat high-carbohydrate (HFHC) meal, an oral lipid tolerance test (OLTT), an intravenous glucose tolerance test (IVGTT), as well as a EHIC). Perturbing metabolic homeostasis by diverse stressors can allow for the observation of an individual's metabolic flexibility, and thus the adaptability to cope with alterations in the supply of energy from a given substrate. The metabolic challenges set for the apparently healthy cohort were aimed at elucidating early metabolic dysregulation associated with FTO obesity risk variants, preceding the onset of disease (Wahl et al. 2014). Whilst plasma metabolic profiles in response to the HFHC, OLTT, IVGTT and EHIC dietary stimuli were not different between FTO genotypes, there was a minor genotypic effect in response to the OGTT, with the AA genotype associated with decreased levels of plasma phospholipids (Wahl et al. 2014). This may suggest a difference between allelic variants of FTO on phospholipid clearance in response to insulin (Wahl et al. 2014).

It is important to note that plasma can be influenced by factors other than the skeletal muscle (i.e. the liver) and thus may not necessarily provide any indication of the metabolic influence of skeletal muscle. To date, no published metabolomics approach has investigated whether FTO genotype specific differences in metabolic profiles exist in skeletal muscle tissue.

2.6.3 The Impact of Exercise on FTO

Metabolic perturbations secondary to exercise may be responsible for attenuating the influence of FTO on obesity susceptibility. Studies investigating human cohorts from high income countries have shown the association of FTO variants, including

rs9939609, and obesity related phenotypes (including body fat accumulation and waist circumference) to be more prominent in those who have physically inactive lifestyles, with this association weakening in individuals who are physically active (Andreasen et al. 2008; Lee et al. 2010; Rampersaud et al. 2008; Vimaleswaran et al. 2009). Additionally, a meta-analysis encompassing 45 studies provides support for a gene-lifestyle interaction between FTO and physical activity, with the association between the rs9939609 risk A-allele and the odds of obesity attenuated by approximately 30% in physically active adults compared to those who are physically inactive (Kilpeläinen et al. 2011). It should be acknowledged that despite the bulk of data supporting a gene-lifestyle relationship between FTO variants and physical activity on obesity related phenotypic outcomes, contradictory data does exist. Physical activity did not modify the influence of the FTO rs9939609 polymorphism on obesity risk in a cohort of healthy adults from high-income countries (Jonsson et al. 2009), or in obese male adults (Berentzen et al. 2008). Additionally, no association between FTO variants and markers of physical activity has been indicated in children (Hakanen et al. 2009; Kilpeläinen et al. 2011; Wardle et al. 2009). Inconsistencies may be due to variable classifications of physically activity, and/or due to different methodologies in statistical analysis and result presentation (Kilpeläinen et al. 2011; Palla et al. 2010). The self-reporting nature of much of data collated in gene-lifestyle interaction investigations may also account for inconsistencies between studies. Due to the aforementioned factors, it is also possible that a gene-lifestyle interaction between FTO and physical activity may be either under- or overestimated. If exercise can attenuate FTO's influence on obesity risk, then exercise may impact the function of the FTO protein in the skeletal muscle. Research into this area is thus far unexplored, and clearly warranted.

2.7 Cellular Functions of FTO

GWA studies and metabolic marker investigations have provided valuable clues and direction as to FTO's potential influence on metabolism. However, studies that also investigate cellular functions of FTO remain fundamental, as functional processes at a cellular level can impact phenotypic outcomes (Barrès et al. 2012). Although FTO is ubiquitously expressed, it is relatively lower in peripheral tissues than in the central nervous system (Gerken et al. 2007; Stratigopoulos et al. 2008). The function of the FTO protein may be multifaceted due to its subcellular distribution. Within the cell, FTO resides in both the nucleus (a location enriched with mRNA) and cytoplasm (a location where metabolic pathways occur, including glycolysis) (Gulati et al. 2014). A binding partner of FTO (Exportin 2, XPO2) has been proposed to be involved in shuttling FTO between the two compartments (Gulati et al. 2014). The FTO protein also has a partial co-localisation with nuclear speckles (splicing factors) (Berulava et al. 2013; Jia et al. 2011), which are involved in RNA translation machinery (Spector & Lamond 2011). This implicates FTO in multiple cellular functions, including RNA processing (Berulava et al. 2013; Jia et al. 2011). Additionally, FTO has been suggested to function as a nutrient sensor, with links to glucose and amino acid availability (Cheung et al. 2013) as well as the signalling of mTORC1 (a key regulator of cell growth and translation) (Gulati et al. 2013). The following section summarises what is known regarding the cellular functions of FTO via investigations of the transcriptome and proteome, including the FTO proteins role as a nucleic acid demethylase, and how these may influence metabolic outcomes.

2.7.1 FTO mRNA and Protein Expression

Exercise training stimulates adaptive responses to improve metabolic efficiency, oxidative capacity, and contractile activity through the alteration of gene and protein expression levels (see Coffey & Hawley 2007 for a detailed review). The ability for exercise to activate a complex array of transcriptional changes in target tissues is important for physical health, weight maintenance, and can have beneficial effects on markers of metabolic syndrome (Rönn et al. 2013). Whether these cellular adaptations are influenced by FTO variants in response to varying exercise intensities is unknown. However, previous investigations have examined FTO protein expression and/or transcription in various tissues in basal, fasted or nutritionally stimulated states.

FTO mRNA and protein expression in basal (resting) skeletal muscle has been shown to be significantly elevated in patients with T2DM in comparison to age-matched, healthy lean controls (Figure 2.4 A & B) (Bravard et al. 2011). On the contrary, obese and type 1 diabetic patients exhibited similar expression levels FTO mRNA and protein, when compared to that of a lean control group (Bravard et al. 2011). Thus, elevated FTO mRNA and protein expression in skeletal muscle are seemingly related to the disease state of T2DM, rather than the passive state of obesity. Interestingly, no relationship between FTO mRNA or protein expression and insulin sensitivity were observed, and neither insulin or glucose infusions (via clamp methods) were found to regulate FTO mRNA expression levels in any of the aforementioned participant subgroups (Bravard et al. 2011). Bravard and colleagues (2011) did not explore the influence of insulin and glucose infusions on FTO protein expression levels. Furthermore, genotypic profiling was not conducted by Bravard and colleagues (2011), thus it is unknown whether differences across genotype alleles exist for the

elevated levels of FTO mRNA and protein expression observed in T2DM patients, or for the expression levels observed in the other studied subgroups.

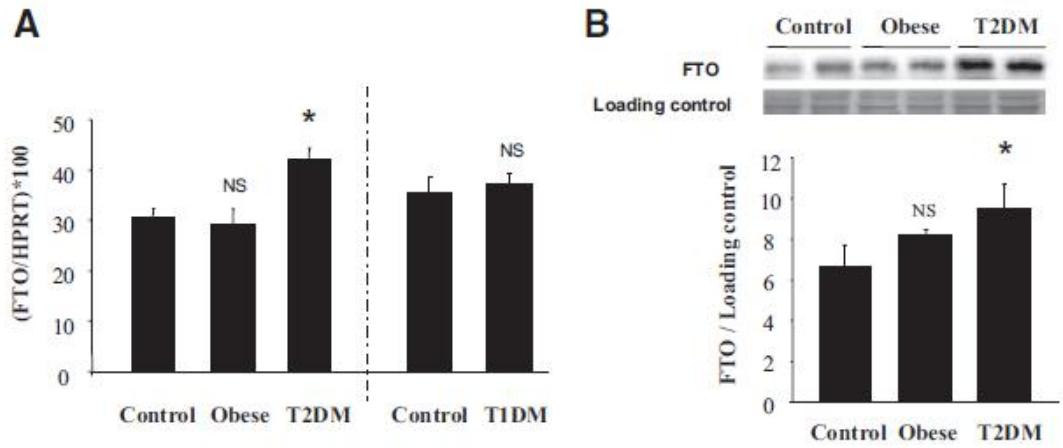


Figure 2.4 A) Skeletal muscle FTO mRNA expression in obese, type 1 and 2 diabetic patients, and age-matched lean control participants at rest. B) Skeletal muscle FTO protein expression in obese, type 2 diabetic patients, and controls at rest. * $p < 0.05$ v. control. Reproduced from Bravard et al. 2011.

Human investigations have indicated skeletal muscle FTO mRNA expression to be both associated (Grunnet et al. 2009*b*), and not associated (Grunnet et al. 2009*a)*, with substrate oxidation in response to metabolic stimuli via differing metabolic models. Whilst Grunnet et al. (2009*a*) reported no correlation between these factors either prior to or following an IVGTT, a follow up investigation by the same laboratory group showed FTO mRNA expression to be negatively associated with fat and positively associated with glucose oxidation rates in response to a EHIC (Grunnet et al. 2009*b*). Additionally, an age-dependent decline in FTO mRNA expression was found to be associated with peripheral defects in glucose and lipid oxidation, independent of fat mass (Grunnet et al. 2009*b*). Furthermore, a positive correlation between FTO mRNA expression and the mRNA expression of genes involved in

energy metabolism, in particularly peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC1α) and glucose transporter type 4 (GLUT4), were also observed in response to the EHIC (Grunnet et al. 2009*b*). This finding could indicate that FTO expression levels in the skeletal muscle may play a role in components of metabolic syndrome, namely peripheral metabolic regulation (Grunnet et al. 2009*b*). Additionally, these results may indicate that like PGC1α and GLUT4, FTO may also be a nutritionally regulated metabolic gene. The role of metabolic genes is to control metabolism by restoring metabolic functions upon environmental changes. This notion has since been supported in chickens, with the consumption of a high glucose diet markedly increasing skeletal muscle FTO and PGC1α mRNA expression (Jia et al. 2012). The association detected in Grunnet and colleagues second human study (2009*b*), in comparison to that of the groups previous work (2009*a*), may have been facilitated by investigating a larger cohort (158 v. 46 skeletal muscle samples), the use of twin participants to enhance homogeneity, and methodological differences in the chosen metabolic stimuli techniques (EHIC v. IVGTT). Both investigations also examined the influence of the FTO rs9939609 polymorphism on FTO mRNA expression and found no genotypic difference in either its transcription in the skeletal muscle or its relationship with substrate oxidation (Grunnet et al. 2009*a*; Grunnet et al. 2009*b*). Currently these are the only two human studies that have investigated skeletal muscle FTO mRNA expression between rs9939609 genotypes, with no published investigation to date having examined whether differences between allelic variants of this polymorphism exist for skeletal muscle protein expression. Furthermore, the findings by Grunnet and colleagues (2009*a* & 2009*b)* do not exclude an influence of FTO genotype on the function of its protein, in terms of enzymatic activity.

FTO mRNA has been shown to be expressed at greater levels in the peripheral blood cells of healthy lean individual's homozygous for the rs9939609 risk A-allele compared to those who had not inherited the A-allele (TT genotypes) (Berulava & Horsthemke 2010; Karra et al. 2013). An increased expression of FTO mRNA in peripheral blood cells is not likely to be involved in the regulation of body weight alone (Berulava & Horsthemke 2010), and interestingly, AA genotypes were also found to have a 2.5-fold increase in the peripheral blood cell mRNA expression of ghrelin compared to their TT genotype counterparts (Karra et al. 2013). Ghrelin is a gut hormone that is released in response to nutrient ingestion and visual cues (via a psychosomatic response), and plays a fundamental role in appetite and body weight regulation (Cummings & Overduin 2007; Karra et al. 2013). Perturbations to the mechanism of ghrelin production and release may offer a biologically plausible explanation to the well-documented obesity-prone eating behaviours of individuals homozygous for the FTO rs9939609 risk A-allele, including documented increases in daily energy intake in comparison to TT genotypes (Speakman et al. 2008; Tanofsky-Kraff et al. 2009; Timpson et al. 2008; Wardle et al. 2008; Wardle et al. 2009).

The level of genetically engineered *Fto* overexpression in mice is associated with a dose dependent increase in total mass and fat mass, in addition to an enhanced food intake (when compared to wild-type mice) (Church et al. 2010). Furthermore, an overexpression of the FTO protein in myotubes results in increased basal protein kinase B phosphorylation, enhanced lipogenesis, oxidative stress, and a reduction in mitochondrial oxidative function (Bravard et al. 2011). These metabolic alterations demonstrate strong similarities to those previously observed in individuals who have T2DM, with diabetic skeletal muscle characterised by increased lipid accumulation

(Kelley & Simoneau 1994) and oxidative stress (Bonnard et al. 2008). This may suggest that an increased expression of the FTO protein may contribute to the muscle defects that characterise T2DM; a theory supported by the elevated FTO protein expression observed in the skeletal muscle of individuals with T2DM (Bravard et al. 2011). Furthermore, FTO protein overexpression in myotubes results in a significant reduction of the iron sulfur subunit of mitochondrial complex II at the protein level (Bravard et al. 2011). Complex II plays an important role in energy production, functioning in the TCA cycle by oxidising succinate to fumarate and in the mitochondrial electron transport chain (ETC) by transferring electrons to ubiquinone (Coenzyme Q10 in its oxidised form) (Jain-Ghai et al. 2013). Whilst defects in mitochondrial complexes may lead to a reduction in adenosine triphosphate (ATP) production, ATP synthesis mediated by complex II substrates was not modified by (Bravard et al. 2011). Additionally, mice with a partial loss of *Fto* function have elevated skeletal muscle mRNA expression of genes involved in carbohydrate metabolism (including glucose-6-phosphate dehydrogenase (G6PD), pyruvate dehydrogenase kinase (PDK4) and insulin receptor substrate 1 (IRS1)) in comparison to control littermates (Church et al. 2009). The up regulation of these genes supports the notion that skeletal muscle metabolism may be altered by the *Fto* protein (Church et al. 2009).

Cheung et al. (2013) utilised an *in vitro* approach to examine whether nutrients could regulate the expression of FTO, with glucose and essential amino acid deprivation of hypothalamic cells (both mouse and human) able to reduce the expression of FTO mRNA and protein. This downregulation did not occur with nonessential amino acid deficiency (Cheung et al. 2013). The replacement of amino acids was found to reverse

the deprivation effect on FTO expression levels, suggesting that nutrient sensing of essential amino acid availability may be a mechanism through which FTO influences energy availability and metabolism (Cheung et al. 2013). Moreover, mTORC1, a key cell growth and translation gene, can be influenced by the enzymatic activity of FTO, thus providing further evidence for a role of the FTO protein in nutrient sensing (Gulati et al. 2013). A downregulation of FTO protein expression deprives a cell of the ability to respond appropriately to amino acids, consequentially causing mTORC1 signalling to become deregulated, even in the presence of amino acids (Gulati et al. 2013). A reduction in mTORC1 signalling increases autophagy, the catabolic mechanism that maintains cellular energy levels and ensures cell survival during starvation through the degradation of unnecessary or dysfunctional cellular components (Gulati et al. 2013). As skeletal muscle tissue is the largest depot of protein in the body, it would presumably be highly sensitive to a sudden downregulation of FTO and subsequent increase in autophagy, explaining the dramatic loss of lean muscle mass observed in *Fto* knockout mouse models (Fischer et al. 2009; McMurray et al. 2013). This relationship between FTO and metabolism is further supported by Merkestein and colleagues (2014), with an up regulation of cellular anabolic growth pathways and concurrent downregulation of catabolic pathways observed when overexpressing the *Fto* protein in mice.

The liver is a highly metabolic tissue that may respond rapidly to energy states, providing potential clues on the peripheral influences of FTO. Poritsanos et al. (2010) was the first to suggest that the *Fto* protein may participate in the regulation of glucose metabolism via gluconeogenesis (the process by which glucose is generated via non-carbohydrate sources) within the liver. Specifically, *Fto* protein deficient

mice showed positive correlations between hepatic *Fto* mRNA expression and the hepatic mRNA expression of glucose-6-phosphatase (G6PC) (a rate limiting gluconeogenic enzyme) in response to an intraperitoneal glucose injection (Poritsanos et al. 2010). Hepatic *Fto* expression was also influenced by nutrients/metabolic stimuli, with the aforementioned insulin stimulated state associated with a reduced hepatic FTO mRNA expression in *Fto* protein deficient mice, whilst an elevation in hepatic FTO mRNA occurred as an effect of fasting in these models (Poritsanos et al. 2010).

A relationship between FTO and gluconeogenesis was recently verified *in vitro,* with FTO protein overexpression in chicken embryo fibroblast cells resulting in significantly lower glucose concentrations in cultured media, when compared to that of control cells (Guo et al. 2015). The overexpression of FTO in these cells was also associated with an increase in the mRNA expression, protein expression and enzymatic activity of G6PC, with the opposite effect on G6PC observed in FTO knockdown cells (Guo et al. 2015). It is expected that an increased G6PC expression and activity would be associated with elevated glucose concentrations in the media, rather than decreased. Thus, it was speculated that this observation may be due to FTO promoting cellular glucose uptake independently of gluconeogenesis (Guo et al. 2015). Guo et al. (2015) also proposed that glucose losses caused by an increased cellular uptake may have exceeded the glucose gain from gluconeogenesis, and/or that an increase in gluconeogenesis may have been a physiological response to the lower glucose concentration in the media resulting from FTO protein overexpression enhancing cellular glucose uptake. Additionally, mice with hepatic overexpression of the *Fto* protein develop hyperglycemia, hyperinsulinemia and glucose intolerance,

supporting a potentially novel role of FTO in the regulation of glucose metabolism *in vivo* (Bravard et al. 2014).

These observations in glucose metabolism indicate a metabolic role of *Fto* in peripheral tissues. Meanwhile, the effects of nutritional status on *Fto* protein expression in the hypothalamus is equivocal (Fredriksson et al. 2008; Gerken et al. 2007; McTaggart et al. 2011; Wang et al. 2011). Research has shown 48-h fasting in mice and long-term food restriction in rats to decrease FTO protein expression in the hypothalamus (Gerken et al. 2007; Wang et al. 2011), whilst conversely, 48-h fasting in rats was reported to increase *Fto* protein expression in this tissue (Fredriksson et al. 2008). Furthermore, an additional animal investigation exploring hypothalamic *Fto* protein expression reported no changes when using similar a dietary stressor (McTaggart et al. 2011). It is likely that variations in *Fto* expression outcomes may reflect differences in experimental techniques, including the model strains used and whether the whole hypothalamus or individual regions of the hypothalamus were investigated (McMurray et al. 2013). At the level of mRNA, Poritsanos et al. (2011) found fasting to reduced *Fto* transcription in the hypothalamus of wild-type mice, whilst the effect of fasting was reversed with intraperitoneal glucose treatment. Furthermore, intracerebroventricular administration of glucose treatment increased hypothalamic *Fto* mRNA levels in fasted mice (Poritsanos et al. 2011). Conversely, obesity was associated with impairments in glucose-mediated regulation of hypothalamic *Fto* mRNA expression, with researchers concluding that hypothalamic *Fto*-expressing neurons may have a role in the regulation of metabolism by monitoring metabolic states of the body (Poritsanos et al. 2011).

2.7.2 Epigenetic Effects of FTO

As previously mentioned, FTO belongs to the AlkB family of Fe^{2+}- and 2OG-dependent oxidative DNA and RNA demethylases (Gerken et al. 2007). 2OG (also commonly referred to as alpha-ketoglutarate) is a co-substrate of FTO, a key metabolite of the TCA cycle (which is responsible for aerobic metabolism in the skeletal muscle), and a marker of nutritional status (Ma et al. 2012). 2OG oxygenases are involved in a range of diverse processes, including DNA repair and fatty acid metabolism (Gerken et al. 2007). Thus, the catalytic activity of FTO may regulate the transcription of genes involved in metabolism via nucleic acid demethylation. Within the TCA cycle 2OG is produced by the decarboxylation of oxalosuccinate, after which, 2OG is decarboxylated further to form succinyl-CoA. 2OG oxygenases are also involved in the production of succinate, formaldehyde and carbon dioxide in the TCA cycle (Gerken et al. 2007). Despite this, it is unlikely that FTO directly influences cellular metabolism via this metabolite, as kinetic analysis has shown the 2OG K_m of FTO to be 2.88 $\mu mol.L^{-1}$, which is at least 10-fold lower than the intracellular concentration of 2OG (50-100 $\mu mol.L^{-1}$) (Ma et al. 2012). As enzymatic activity would depend on the physiological range of 2OG concentrations, it is unlikely that FTO would be either a sensor for intracellular 2OG or able to regulate any physiologically relevant 2OG dependent systems within the TCA cycle (Ma et al. 2012). However, emerging evidence has indicated energy metabolism alterations within the TCA cycle to modulate the epigenetic programming of the genome, thereby also influencing gene expression. Specifically, fumarate and succinate have been indicated as inhibitors of 2OG activity (Gerken et al. 2007; Xiao et al. 2012). Furthermore, an accumulation of these metabolites can induce hypoxic conditions associated with an elevated transcription of hypoxia inducible factor 1 (HIF-1α),

whilst simultaneously inhibiting the activity of several DNA demethylases (Selak et al. 2005; Xiao et al. 2012). It is therefore proposed that conditions that can elevate fumarate and succinate levels, such as exercise (Gibala et al. 1997*a*; Gibala et al. 1997*b*), may be able to modulate FTO activity. Furthermore, the downregulation of FTO function by these TCA cycle intermediates supports a role of the FTO protein in energy pathways (Gerken et al. 2007).

A known function of the FTO protein is its ability to catalyse the demethylation of 3-meT and 3-meU on single stranded DNA and RNA, respectively (Gerken et al. 2007; Han et al. 2010). A more recently identified role of the FTO protein is its ability to demethylate m^6A on RNA (Jia et al. 2011), which is an indication of a developmentally and dynamically regulated post-transcriptional modification (Meyer et al. 2012) that is reversible via the actions of methyltransferases and demethylases (Niu et al. 2013) (Figure 2.5). This is key to the potential regulatory role of FTO, with RNA considered a primary substrate of FTO due to 1) the high abundance of RNA in cells, and 2) the FTO proteins strong affinity for single stranded RNA (Gerken et al. 2007; Han et al. 2010). Additionally, FTO has been associated with methylation changes within itself, in addition to several other genes, suggesting that the effect of FTO on obesity predisposition may, at least in part, be mediated through this epigenetic alteration (Almén et al. 2012). Epigenetics is defined as "the structural adaptation of chromosomal regions so as to register, signal or perpetuate altered activity states" (Bird 2007). The epigenetic state of human DNA, and the related phenotype, can be inherited in what is referred to as transgenerational epigenetic inheritance (Morgan & Whitelaw 2008). Furthermore, epigenetic profiles and resultant phenotypic traits can be influenced by age dependent alterations including

environmental, behavioural, psychological and pathological stimuli (Fraga et al. 2005; Rönn et al. 2013), and these alterations may also be transmitted down the cell lineage or across generations (Carone et al. 2010). Epigenetic alterations assumed to be unrelated to inheritance may also occur throughout adulthood, and are typically triggered by acute environmental influences, such as exercise (Barrès et al. 2012; Nitert et al. 2012). There are three main epigenetic mechanisms: 1) methylation, 2) histone modification, and 3) the modulation of gene transcription and translation by non-coding RNA's (or microRNAs (miRNAs)) (Kirchner et al. 2013). These epigenetic mechanisms have been well documented to play a fundamental role in the regulation of both physiological and pathophysiological outcomes in humans (Egger et al. 2004; Portela & Esteller 2010). This review will focus on the epigenetic process of methylation.

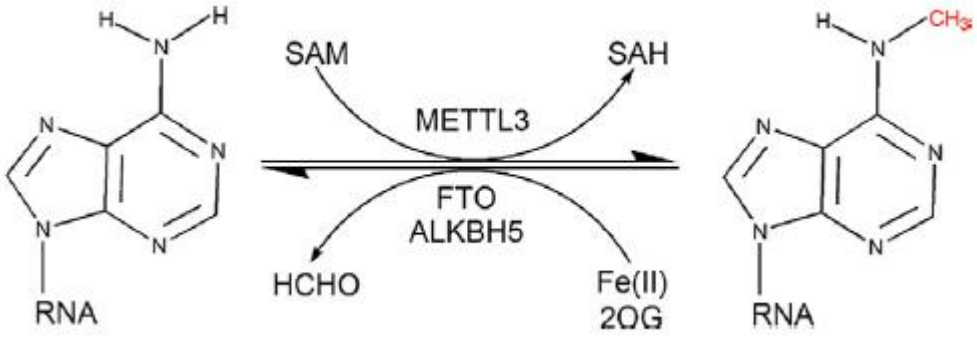


Figure 2.5 Reversible m^{6}A methylation in mRNA. The m^{6}A modification is catalysed by a methyltransferase complex containing methyltransferase like 3 (METTL3) with S-adenosylmethionine (SAM) as a methyl donor for m^{6}A formation. AlkB family member 5 RNA demethylase (ALKBH5) and FTO demethylate m^{6}A in an iron and α-ketoglutarate-dependent manner. Reproduced from Niu et al. 2013.

2.7.2.1 DNA Methylation

DNA methylation influences gene activity by silencing genes and DNA regions in which transcription is not desired (Curradi et al. 2002). This epigenetic modification can be established early in life (through maternal dietary exposure) and maintained in differentiated cells, therefore making DNA methylation a likely determinant of phenotypic outcomes in adulthood (Waterland & Jirtle 2003). On such a basis, DNA methylation is proposed to play an important role in the development and predisposition of metabolic disease states by modifying the expression of genes controlling whole body energy and glucose homeostasis (Barrès et al. 2009; Klose & Bird 2006).

There is indication that the *Fto* protein could be a transcriptional coactivator that can enhance the binding of CCAAT-enhancer binding protein beta (CERPβ), a key transcriptional regulator of adipogenesis (Wu et al. 2010). Thus, *Fto* may exert a role in the epigenetic regulation of CERPβ, the development and maintenance of fat tissue, and subsequent obesity development, through the modulation of energy homeostasis (Wu et al. 2010; Zhao et al. 2014). Furthermore, genome wide methylation profiling studies have previously indicated that the passive state of obesity itself can contribute to the DNA methylation of several genes (Almén et al. 2012; Wang et al. 2010), and that DNA methylation status can predict weight loss outcomes (Milagro et al. 2011).

Methylation status is potentially genotype specific, and as such genetic variations may regulate epigenetic changes (Bell et al. 2010; Heijmans et al. 2007). Findings from human peripheral blood analysis have suggested that allelic variants of the FTO rs9939609 polymorphism may mediate the DNA methylation status (both hyper- and

hypomethylation) of multiple genes, independent of BMI (Almén et al. 2012). Five genes (lysyl-tRNA synthetase (KARS), telomeric repeat-binding factor 2-interacting protein 1 (TERF2IP), dexamethasone-induced protein (DEX1), musashi 1 (MSI1) and breast carcinoma amplified sequence 3 (BCAS3)) showed significantly greater methylation levels in AA genotypes compared to TT genotypes, whilst one gene (stonin 1 (STON1)) was significantly lower (Almén et al. 2012). Greater methylation of KARS and TERF2IP in AA genotypes was speculated by Almén et al. (2012) to potential occur secondary to structural alterations of chromosome 16 (where they both reside in addition to FTO), and/or due to a regulatory response of the FTO protein to maintain homeostasis between protein complex subunits. No clear connection for the remaining five differentially methylated sites and FTO were drawn. Albeit, whilst various states of DNA methylation can exist with differences in FTO genotype, it is unknown whether this leads to subsequent translational or enzymatic differences.

2.7.2.2 RNA Methylation

mRNA is a critical link in the flow of genetic information from DNA to functional protein, and thus, a key regulator of various biological processes (Wang et al. 2014). The reversible and dynamic methylation of mRNA provides a highly sophisticated regulatory system capable of influencing gene expression (Fu et al. 2014; Wang et al. 2014). m^6A was first discovered in the 1970's (Desrosiers et al. 1974) and this ubiquitous modification on mRNA accounts for more than 80% of all base coding RNA (including (transfer) tRNA, (ribosomal) rRNA and (small nuclear) snRNA) (Jia et al. 2013; Niu et al. 2013; Pan 2013). The relative abundance of m^6A on mRNA transcripts can impact RNA metabolism processes, such as mRNA splicing, nuclear export, translation efficiency and stability (Jia et al. 2013; Pan 2013) and may act as

an epigenetic modifier of protein expression. Despite this post-transcriptional modification being discovered four decades ago (Desrosiers et al. 1974), it wasn't until recently that FTO became the first enzyme identified to have an ability to reverse m^6A modifications on RNA (Jia et al. 2011), thus demonstrating a novel role of the FTO protein as an epitranscriptomic marker.

Research has shown FTO knockdown (with siRNA) to result in increased m^6A on mRNA in human cells (Jia et al. 2011). Furthermore, FTO knockdown in mice is associated with m^6A hypermethylation of several genes, which concurrently renders their mRNA expression either unchanged or up-regulated, and decreases their protein expression (Hess et al. 2013). This supports a role of FTO activity and m^6A status in translational regulation (Hess et al. 2013). However, the influence of FTO as an m^6A demethylase may be tissue dependent and specific to functionally distinct transcripts as methylation and expression changes did not occur for all genes investigated by Hess and colleagues (2013).

Conversely, the level of m^6A on RNA has been shown to be decreased in cells overexpressing FTO (Jia et al. 2011; Zhang et al. 2015). However, overexpression of the *Fto* protein in mice showed no effect on the m^6A methylation status of either total RNA or total mRNA in skeletal muscle (Merkestein et al. 2014). This finding does not exclude the potential for an influence of FTO on m^6A methylation to be gene specific and/or involve a particular subset of mRNAs. Pathway analysis has revealed methylation pathways to be significantly represented in a subset of genes which exhibited increased expression levels in the skeletal muscle of mice overexpressing FTO (Merkestein et al. 2014). This includes a pathway of methyl transferases and

enzymes involved in the biosynthesis of S-adenosylmethionine (SAM, a major biological methyl donor in cellular processes) (Merkestein et al. 2014). This finding was suggested by Merkestein et al. (2014) to be a compensatory mechanism to counteract the potential demethylation effect of FTO overexpression.

Overexpression of FTO in a mouse cell line has been shown to lead to an elevated abundance of ghrelin mRNA, a reduction in its m^6A methylation status, and ultimately an elevated total ghrelin concentration (Karra et al. 2013). Additionally, peripheral blood cells collected from a lean human cohort has shown AA genotypes of the FTO rs9939609 polymorphism to exhibit increased FTO mRNA expression, greater m^6A ghrelin mRNA demethylation, and a concurrent increase in the expression of this hunger hormone, in comparison to TT genotype counterparts (Karra et al. 2013). This seemingly direct link between FTO and ghrelin in peripheral blood cells provides insight into the epitranscriptomic effects of FTO rs9939609 allelic variants on obesity predisposition. Furthermore, this highlights the need to commence investigations that examine the potential genotype specific metabolic and cellular consequences of the demethylase function of FTO in peripheral tissues, including the skeletal muscle.

Abnormal m^6A methylation levels may lead to dysfunctional RNA metabolism and exacerbate disease processes (Niu et al. 2013). Under heat shock stress (42°C for 1-h) the m^6A demethylase function of FTO is limited by YTH m^6A RNA binding protein 2 (YTHDF2) (a m^6A 'reader' also known as the high-glucose-regulated protein 8), whilst FTO protein expression levels remain unchanged (Zhou et al. 2015). The suppression of the demethylase function of FTO occurs with a heat stress induced

methylation of the 5' untranslated region (5'UTR) on mRNA (Zhou et al. 2015). This not only expands the breadth of physiological roles of m^6A, but also highlights a translational control mechanism in heat shock response (Zhou et al. 2015).

2.7.2.3 Epigenetic Flexibility

Methylation patterns of specific genes are not necessarily global within one organism, and can vary greatly between different cell types within one tissue, or within different tissues of the same individual (Lister et al. 2009; Ollikainen et al. 2010). Methylation status and patterns can also alter with aging (Fraga et al. 2005). Epigenetic alterations to environmental factors tend to be flexible and dynamic, facilitating rapid adaptations to meet acute tissue specific needs, for example, in response to acute external influences such as exercise (Barrès et al. 2012).

Exercise has previously been used as an acute physiological stressor to stimulate epigenetic modifications (including hyper- and hypomethylation) in skeletal muscle tissue (Barrès et al. 2012; Nitert et al. 2012). Furthermore, exercise elicited changes to gene expression can trigger structural and metabolic adaptations (Barrès et al. 2012). Barrès et al. (2012) found a decrease in the DNA methylation status of multiple skeletal muscle specific promotor genes involved in mitochondrial function (including PGC1α, mitochondrial transcription factor A (TFAM), myocyte enhancer factor 2A (MEF2A), and pyruvate dehydrogenase kinase isozyme 4 (PDK4)) following a single bout of high intensity exercise (at 80% VO_{2peak}), whilst the mRNA expression of these genes increased (Barrès et al. 2012). Due to the observed DNA demethylation, Barrès and colleagues (2012) proposed that a single bout of exercise might produce a subcellular response that could potentially counteract the development of metabolic

diseases such as T2DM, which typically exhibit an elevated methylation status of the aforementioned mitochondrial genes. Interestingly, whilst acute high intensity exercise was found to influence skeletal muscle DNA methylation, low intensity exercise did not, suggesting a dose-dependent effect of exercise intensity on DNA methylation (Barrès et al. 2012).

The use of exercise to explore rapid and adaptive epigenetic modifications triggered by physiological and metabolic perturbations may help further investigate the cellular mechanisms of disease susceptibility genes, including FTO. Additionally, if metabolic health can be improved via the flexible epigenetic mechanisms produced by lifestyle modification, then this may help to explain why physical activity attenuates the odds of obesity in FTO risk A-allele carriers. No published study to date has examined whether differences in exercise-induced epigenetic modifications exist between allelic variants of the FTO rs9939609 polymorphism.

2.8 Further understanding the influence of FTO

Although FTO harbours the strongest known susceptibility locus for obesity, the exact biological mechanisms by which its different allelic variants influence obesity predisposition remain unclear. Due to the substantial socioeconomic burden of obesity, research that deepens our understanding of genetics in relation to the complex regulatory mechanisms governing energy homeostasis is necessary. This includes expanding our knowledge on the mechanisms of FTO, particularly in regards to its metabolic and functional influences. We know that FTO is expressed in specific areas of the brain, skeletal muscle, and adipose tissue, and it is due to the ubiquitous nature of FTO that there is an added complexity in associating disease susceptibility polymorphisms to specific cellular functions in order to explain previously established clinical associations. Bioinformatic analyses has shown FTO to demethylate m^6A on RNA (Jia et al. 2011), as well as 3-meT and 3-meU on single stranded DNA and RNA, respectively (Gerken et al. 2007; Jia et al. 2008). Human and mouse model investigations have found m^6A at specific mRNA sites to perform a fundamental role in the regulation of gene expression, as well as exerting varying effects on mRNA splicing and transport (Dominissini et al. 2012; Meyer et al. 2012). Changing expression levels of the FTO protein potentially alters the m^6A landscape, possibly influencing rates of RNA translation (Gulati et al. 2013). How the demethylation activities of FTO may be integrated into the complex network of energy metabolism control remains to be elucidated (Müller et al. 2013).

Based on current knowledge, the FTO protein is critical for life, and plays a role in the regulation of energy metabolism and intake (Church et al. 2009; Church et al. 2010; Fischer et al. 2009). A heightened risk of developing obesity and associated disease

(i.e. T2DM) is linked to an increased expression of FTO (Bravard et al. 2011; Church et al. 2010). Whilst the effects of FTO in the central nervous system are well studied, research into FTO's peripheral effect (particularly within the skeletal muscle) is limited. Specifically, it is unclear how the potential influence of elevated FTO expression on disease risk is mediated in the skeletal muscle. In this review, it has been speculated that exercise may be able to impact FTO protein function, as this could explain the apparent ability of physical activity to attenuate FTO's influence on obesity susceptibility. However, it is not known if or how this may occur, or whether exercise stimulated metabolic influences may facilitate this potential effect. The use of metabolic stimuli and identification of biomarkers that characterise genotype specific metabolic and cellular changes within human skeletal muscle may assist in uncovering the mechanisms by which FTO influences obesity predisposition. To date previous literature has shown little differences between allelic homologues of the FTO rs9939609 polymorphism in various forms of measurements at rest and in response to nutritional stimuli (Grunnet et al. 2009*a*; Kjeldahl et al. 2014; Wahl et al. 2014). Investigations that perturb energy flux via exercise may assist in unmasking the function of the FTO protein in peripheral tissues. However, researchers have not yet used exercise stimuli to examine whether differences between allelic variants exist for metabolic profiles in the skeletal muscle, or for the expression and function of FTO in this tissue. Additionally, no investigation has examined the influence of the FTO rs9939609 polymorphism on metabolic flexibility (typically measured as ΔRER) in response to acute nutritional stimuli. Previous research has associated T2DM and insulin resistance with impaired metabolic flexibility (Kelley et al. 1999; Kelley et al. 1994), and thus research into whether FTO has a metabolic role in mediating this impairment should also be considered when researching the mechanisms of this gene.

2.9 Aims and Hypotheses

The studies presented in the current thesis are designed to address the gaps in scientific literature to further the knowledge on the metabolic mechanisms of FTO.

1. The aim of the study described in Chapter 4 was to examine if differences in FTO rs9939609 genotype, or BMI, existed in metabolic and respiratory markers of substrate oxidation following an OGL challenge. It was hypothesized that genotypes encompassing the risk A-allele and overweight individuals would display a slower metabolic flexibility compared the TT genotype and lean individuals in response to the OGL.

2. The primary purpose of the study presented in Chapter 5 was to use two acute bouts of isocaloric exercise (at a high and low intensity) to elicit intensity dependent metabolic disturbances in the skeletal muscle and determine the potential of genotype influences on metabolic profiles in this tissue. This project also investigated whether differences between allelic variants existed for substrate utilisation during exercise. It was hypothesized that variations in metabolic profiles would exist between allelic homologues of FTO. It was additionally hypothesized that the ability to adjust substrate utilisation in response to metabolic demand would be reduced in risk A-allele carriers (AA and AT genotypes) compared to their homozygous non-risk allele counterparts (TT genotypes).

3. The aims of Chapter 6 were twofold; Firstly, to measure whether difference in FTO mRNA and protein expression, and m^6A on RNA, exist in skeletal muscle. Secondly, to observe whether an acute bout of exercise influenced FTO expression and function (measured by m^6A methylation status) in the skeletal muscle. It was hypothesized that exercise would induce a greater elevation in skeletal muscle FTO mRNA and protein expression on variants encompassing the risk A-allele (AA and AT genotypes), and increased demethylation of m^6A on RNA, in comparison to individuals homozygous for the non-risk allele (TT genotypes). It was also hypothesized that the higher intensity exercise would induce greater perturbations to FTO expression and function than lower intensity exercise.

4. The investigation described in Chapter 7 was conducted independently as a pilot study to optimise metabolomic specific methodologies in plasma prior to this technology being used to analyse the skeletal muscle samples obtained in Chapter 5. The aim of this study was to compare two workload matched supramaximal low volume HIE models using an untargeted metabolomics approach. It was hypothesized that despite the workload matched nature of the protocols, the higher supramaximal exercise intensity would induce greater metabolic perturbations in glucose metabolism during exercise and fatty acid and lipid metabolism during the recovery period, compared to the lower supramaximal exercise intensity.

CHAPTER THREE

Experimental Methods and Procedures

This chapter describes the general methods and procedures used in the four studies presented in this thesis. The first study used a nutritionally induced metabolic stimulus, via an oral glucose load (OGL) challenge, to investigate whether metabolic flexibility was influenced by FTO genotype. The second and third studies investigated exercise intensity dependent effects on skeletal muscle metabolic profile, as well as FTO expression (mRNA and protein) and FTO protein function, and examined whether a difference between allelic variants existed for these variables. An additional pilot study was conducted to optimise metabolomics techniques, and examine plasma metabolites responses to workload matched supramaximal exercise of varying intensity. Details of study specific experimental procedures are described within the relevant chapter.

3.1 Human Research Ethics Approval

All investigative processes detailed in this thesis had been approved by the Victoria University Human Research Ethics Committee *(HRETH 12/197)* (Chapters 4, 5 and 6) and *(HRETH 10/12)* (Chapter 7) prior to study commencement. All studies were performed in accordance with the ethical standards set out in the 1964 Declaration of Helsinki (WMO 1996). Participants were recruited by electronic and print methods, and were generally students of Victoria University. All participants were informed verbally, and additionally in writing, as to the objective of the experiments, as well as the potential risks associated. Participants believed to meet the eligibility criteria set for each study were asked to provide written consent and complete a medical questionnaire using the appropriate documentation previously approved within each studies respective ethics application.

3.2 Participants

A total of 150 apparently healthy untrained males and females aged between 20 - 50 years were recruited to examine the metabolic mechanisms of the FTO gene. Participants were excluded from participating within the study if they had diagnosed diabetes (fasting blood glucose greater than 7.0 $mmol.L^{-1}$), were performing any regular fitness training (> 30 mins, 3 x per week) for 6 months prior, were taking contraindicated prescription medication that may influence metabolism (including thyroid, hyperlipidmeic, hypoglycaemic, and/or antihypertensive agents), or if they were pregnant. When providing consent to participate in this study all participants selected to partake in the initial trial day only (phase one: OGL challenge, discussed in Chapter 4), or following the initial trial day to continue with a second phase of the

study involving four additional experimental trial days (phase two: HI and LO intensity exercise testing, discussed in Chapters 5 and 6).

Participants were asked to refrain from consuming caffeine and alcohol, and from undertaking strenuous exercise, 24-h prior to attending each of the experimental trials. Participants recorded their dietary intake over 4 days prior to the initial trial day, and were asked to consume a carbohydrate predominant meal the night before this trial. Those continuing into phase two were asked to replicate their dietary intake in the 24-h prior to experimental exercise trial days, in addition to following the aforementioned guidelines (refraining from caffeine, alcohol and exercise). All experimental trials were conducted in the morning, 10 – 12 h after the last meal, to ensure participants were in a preprandial/basal state.

An additional seven apparently healthy untrained male participants aged between 18 – 35 years were recruited for the pilot study described in Chapter 7. Participants were excluded from partaking in this study if they had diagnosed diabetes (fasting blood glucose greater than 7.0 $mmol.L^{-1}$), were performing any regular fitness training (> 30 mins, 3 x per week) for 6 months prior, were taking contraindicated prescription medication that may influence metabolism (including thyroid, hyperlipidmeic, hypoglycaemic, and/or antihypertensive agents), were smokers, or if they had suffered from a muscular or skeletal injury within 6 months prior.

3.3 Genotyping

A standard buccal swab (Illumina, VIC, Australia) from the inside of the cheek was collected to genotype participants for the FTO rs9939609 (T>A) polymorphism. This method required the participant to insert the foam tip of the swab into their mouth and rub it against the inside of their cheek in a circular/back and forth motion for ~20 s. Care was taken to ensure that the foam tip did not touch anything other than the intended surface. Swabs were immediately stored at 4°C for DNA extraction.

QuickExtract DNA solution (Illumina, VIC, Australia) was used to extract DNA from the buccal swabs. This process involved agitating the buccal swab in an eppendorf tube containing 500 µl of QuickExtract solution before heating the sample at 65°C for 1 min, and 95°C for a following 2 mins. These steps were interspersed by 15 s of vortex mixing at 1400 rpm. Following the last heating step samples were centrifuged (1 min at 1400 rpm) and stored at -20°C until quantified for DNA concentration.

Extracted DNA samples were tested for DNA concentration ($ng.\mu l^{-1}$) using a Nanodrop Spectrophotometer (Thermo Scientific, VIC, Australia). Samples were then diluted with nuclease-free H_2O in order to bring them into the appropriate range for subsequent analysis, with the final concentration in each sample being approximately 7 $ng.\mu l^{-1}$ DNA, as confirmed spectrophotometrically.

Genotyping of the rs9939609 (T>A) polymorphism of the FTO gene was performed using the TaqMan allelic discrimination assay (Life Technologies, VIC, Australia). A total of 20 ng of DNA (~2.5 µl per sample) was added to each well of a 96 well plate with 0.5 µl X20 Taqman SNP Genotyping Assay and 5 µl TaqMan Genotyping

Master Mix. Nuclease-free H_2O was then added to each well to create a final well volume of 11 µl. Each sample then underwent 50 amplification cycles using the CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia) under the temperature and time settings detailed in Table 3.1.

Stage	Temperature (°C)	Time	Ramp	Cycles
Pre PCR Read	60	60 s	100	
Holding Stage	95	10 mins	100	
Cycling Stage: **Denature** **Anneal**	 95 60	 15 s 60 s	 100 100	50
Post PCR Read	60	60 s	100	

Table 3.1 Amplification protocol for FTO rs9939609 genotyping using the Taqman SNP genotyping assay and CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia).

For quality control purposes, a positive and negative control was used. The context sequence for the SNP tested was [VIC/FAM]: GGTTCCTTGCGACTGCTGTGAATTT[A/T]GTGATGCACTTGGATAGTCTCTGTT. Fluorescent reporter dye signals of the two probes were monitored throughout the entire amplification using CFX Manager 2.1 software (Bio-Rad Laboratories, VIC, Australia). Allele calling was completed by normalising the end-point to determine relative fluorescence units (RFU), and by then plotting the fluorescent intensity of each probe in an allelic discrimination graph (VIC, y-axis; FAM, x-axis) (Figure 3.1). Genomic 'clusters' were defined manually by sectioning the plot into quadrants with horizontal and vertical lines.

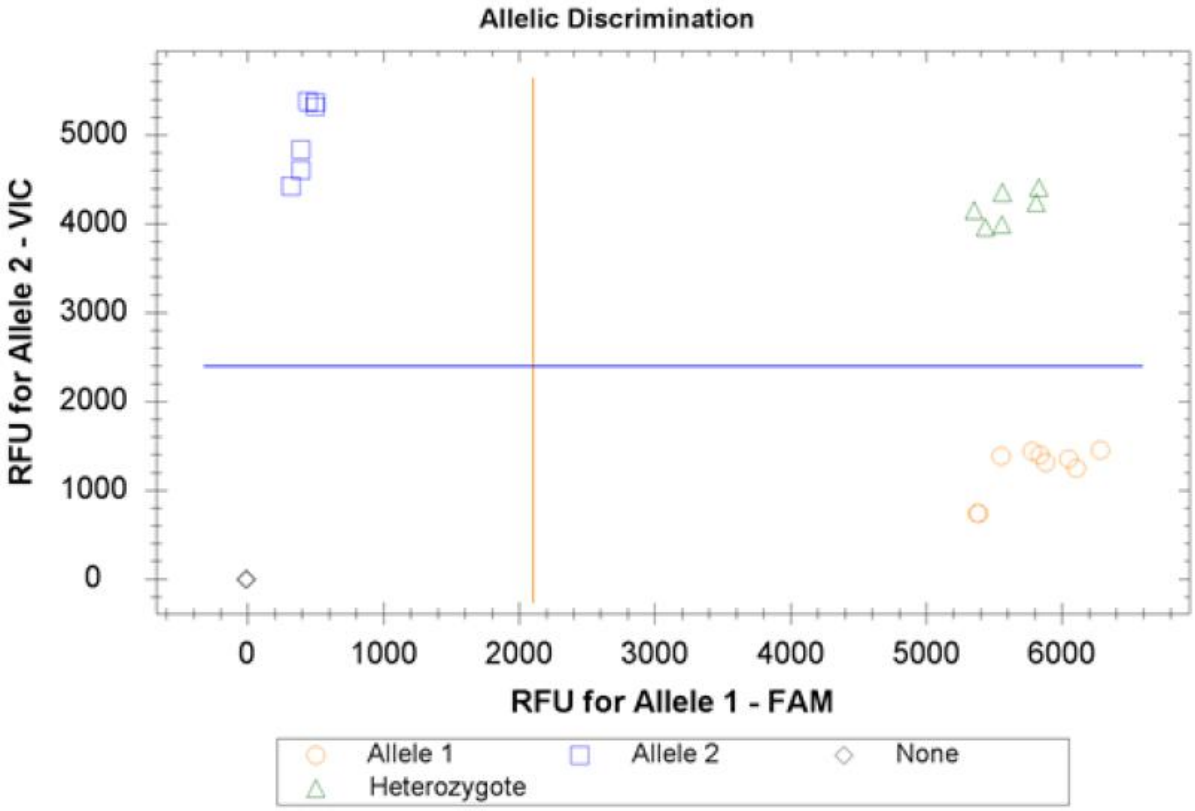


Figure 3.1 Graphic output of the allelic discrimination of FTO by RT-PCR [VIC/FAM] [A/T]. RFU, relative fluorescence units.

3.4 Experimental Trial Protocols

3.4.1 Anthropometric Measurements and Dietary Analysis

Total body mass, fat mass, muscle mass and water mass were recorded using bioelectrical impedance scales (Tanita, VIC, Australia). These scales measure the resistance to an electrical signal as it is circulated through various parts of the body, with muscle and water mass resulting in a less resistant current, and fat mass increasing resistance to the current. Height, hip and waist circumference, and blood pressure were measured using a stadiometer, tape measure and sphygmomanometer (Omron HEM7322, Omron Healthcare, VIC, Australia) respectively. Dietary records (4 day) (see supplementary information for template, Figure S-3.1) were evaluated using Foodworks™ (Australia – Diet and Recipe Analysis (AusFoods), version 7, 2012, Xyris Software) to determine each participants average daily total energy intake, as well as the percentage contributions of protein, carbohydrate, fat, saturated fat (from total fat), alcohol and fibre.

3.4.2 Blood Sampling, Treatment and Analysis

Participants were asked to rest in a supine position whilst a flexible 20-gauge intravenous (I.V.) cannula was inserted into an antecubital vein by a trained phlebotomist. A flow-restricting valve was attached to allow for repeated blood sampling and the I.V. cannula was kept patent using isotonic saline (0.9% NaCl, Pfizer, NSW, Australia).

Approximately 20 mls of venous blood was sampled at various intervals, with study specific time points summarised within the relevant chapter. Each venous blood sample, obtained by drawing from the I.V. cannula with 2 x 10 ml syringes, was placed into lithium heparin and serum-separating tubes (BD Vacutainer, BD Bioscience, NSW, Australia) before being gently inverted and rolled several times. Lithium heparin treated blood was transferred into eppendorf tubes and centrifuged at 12,000 rpm for 2 mins, whilst serum-separating tubes were left to sit at room temperature for 30 mins prior to centrifugation at 1200 G for 10 mins. Plasma and serum samples were decanted and aliquoted into labelled tubes, snap frozen in liquid nitrogen (LN_2), and stored at -80°C for subsequent analysis of various metabolites. An exception to this was plasma samples obtain in Chapter 4, which were analysed immediately for plasma glucose and lactate concentrations (section 3.4.2.1) prior to being snap frozen in LN_2 and stored at -80°C.

3.4.2.1 Plasma Glucose and Lactate Concentration

Plasma glucose and lactate were measured using a Yellow Springs analyser (YSI 2300 STAT, Yellow Springs Instrument, OH, USA). These assays were based on the D-glucose oxidase and L-lactate oxidase analytical principle methods for glucose and lactate respectively (see equations below). The YSI sample chamber aspirated 25 µl of each plasma sample and diffused this across a membrane placed over electrochemical probes containing D-glucose and L-lactate oxidase. D-glucose was oxidised into gluconic acid and hydrogen peroxide (H_2O_2), whilst L-lactate was oxidised to form pyruvate and H_2O_2. H_2O_2 was detected amperometrically at the platinum electrode surface, and hence the current flow at the electrode indicated its concentration. This was directly proportional to the concentration of glucose and lactate present in each sample. All samples were analysed in duplicates with the mean value being used for statistical analysis. The YSI was calibrated after every 5 samples.

Analytical Principle:

$$\text{D-glucose} + O_2 \xrightarrow{\textit{D-Glucose oxidase}} \text{Gluconic Acid} + H_2O_2$$

$$H_2O_2 \xrightarrow{\textit{Platinum anode}} 2H^+ + O_2 + 2e^-$$

$$\text{L-lactate} + O_2 \xrightarrow{\textit{L-Lactate oxidase}} \text{Pyruvate} + H_2O_2$$

$$H_2O_2 \xrightarrow{\textit{Platinum anode}} 2H^+ + O_2 + 2e^-$$

3.4.2.2 Serum Insulin Concentration

Samples were removed from ultra-freeze storage (-80°C) and left to slow-thaw on ice prior to analysis. Serum insulin concentration was determined using an Insulin Enzyme-Linked Immunosorbent Assay (ELISA) Kit (Invitrogen, VIC, Australia). A series of individual insulin standards and controls supplied by the manufacturer were prepared for use by dilution with ultrapure H_2O to produce a final insulin concentration range of 5.1 – 250 $\mu lU.ml^{-1}$.

Standards, controls and serum samples (50 µl) were added in duplicates to the appropriate wells of an insulin antibody-coated 96 well plate followed by 50 µl of anti-insulin horseradish peroxide (HRP) conjugate. Plates were incubated for 30 mins at room temperature whilst being continuously mixed at a low speed. The solution was removed by inversion and the plate tap dried on absorbent paper before 380 µl of 1X wash buffer was added to the wells. Wash buffer was left to soak for 30 s with gentle manual agitation prior to the plate being inverted and tap dried. This procedure was repeated 3 times. Next, 100 µl of stabilised chromogen solution was added to each well to act as a HRP substrate (including to two previously empty wells in order to create a blank for appropriate analysis). The plates were then incubated for 25 mins in the dark at room temperature with continuous mixing at a low speed. To cease the reaction, 100 µl of stop solution was added to each well. The plate was then left to sit in the dark for 1-h prior to the proportion of insulin in each well being determined spectrophotometrically at 450 nm using an X-mark microplate reader (Bio-Rad Laboratories, VIC, Australia). A standard curve was produced using the mean reading for insulin standards minus the mean reading of the blank, in order to determine the insulin concentration (in $\mu lU.ml^{-1}$) of each unknown serum sample.

Pre OGL plasma glucose and serum insulin concentrations were used to calculate the homeostasis model assessment of insulin resistance (HOMA-IR), which is an index of insulin resistance (Matthews et al. 1985):

$$\text{HOMA-IR} = \text{Glucose}^{0} * \text{Insulin}^{0} / 22.5$$

Insulin Sensitivity Index (ISI) was calculated using the composite index that encompasses plasma glucose and serum insulin concentrations sampled pre OGL and at 120 mins post OGL. This method has previously been validated as an index of whole body insulin sensitivity (Matsuda & DeFronzo 1999):

$$\text{ISI (composite)} = 10000 / \text{sqrt} (\text{Glucose}^{0} * \text{Insulin}^{0} * \text{Glucose}^{120} * \text{Insulin}^{120})$$

Plasma glucose was expressed as mmol.L^{-1} and serum insulin as $\mu\text{lU.ml}^{-1}$ during the aforementioned calculations.

3.4.2.3 Plasma Albumin Concentration

Plasma samples were removed from ultra-freeze storage (-80°C) and permitted to slow-thaw on ice pending analysis. A total of 30 µl of each sample was transferred into a labelled corresponding eppendorf prior to adding 30 µl of nuclease-free H_2O (Sigma-Aldrich, NSW, Australia) to create a 2-fold dilution.

Plasma albumin concentration was determined using a Bromocresol Green (BCG) Albumin Assay Kit (Sigma-Aldrich, NSW, Australia), with BCG forming a coloured complex specifically with albumin. A series of albumin standards were prepared from a bovine serum albumin (BSA) stock solution [5.0 $g.dL^{-1}$] by diluting the stock solution with ultrapure H_2O to produce a final BSA concentration range of 0.5 – 5.0 $g.dL^{-1}$.

Blanks, standards and diluted samples (5 µl) were added in duplicates to the appropriate wells of a clear bottom 96 well plate followed by 200 µl of reagent dye. Plates were lightly tapped to mix and avoid bubbles, and sat at room temperature for 5 mins. The proportion of albumin in each well was determined spectrophotometrically at 620 nm using an X-mark microplate reader (Bio-Rad Laboratories, VIC, Australia). A standard curve was produced using the mean reading for albumin standards minus the mean reading of the blank, in order to determine the concentration (in $g.dL^{-1}$) of each unknown diluted plasma sample, then corrected for dilution.

3.4.3 Respiratory Gas Exchange Sampling and Analysis

Respiratory gas exchange was measured using an open-circuit indirect calorimetry metabolic cart (Moxus, AEI Technologies, PA, USA). Expired air was directed by a Hans Rudolph valve via a ventilometer (Moxus, AEI Technologies, PA, USA) into a mixing chamber and analysed for O_2 and CO_2 content (Applied Electrochemistry S-3A O_2 and CD-3A CO_2). Prior to each experimental trial the gas analyser was calibrated using commercially prepared gas mixtures with a range of 16.00 – 20.93% for O_2 and 0.03 – 4.00% for CO_2 (BOC Gases, VIC, Australia). Room condition measurements (temperature, pressure and humidity) were recorded into the gas analyser using a Kestrel 4000 Pocket Weather Tracker (Nielsen Kellerman, ACT, Australia) and volume calibrated using a 3L syringe, prior to each experimental trial.

Participants were fitted with a Hans Rudolph respiratory mouthpiece and nose clip that ensured one-way gas exchange, and had their inspired O_2 and expired CO_2 measured in 30 s intervals (Moxus, AEI Technologies, PA, USA). Energy expenditure was calculated based on the following formula, with respiratory values in $l.min^{-1}$ units:

$$\text{Energy Expenditure } (kJ.min^{-1}) = 16.318*VO_2 - 4.602*VCO_2$$

Substrate utilisation was calculated via commonly used stoichiometric equations (Jeukendrup & Wallis 2005), with the assumption that protein oxidation was minor and constant:

$$\text{Glucose oxidation } (g.min^{-1}) = 4.344*VCO_2 - 3.061*VO_2 - 0.40*n$$

$$\text{Fat oxidation } (g.min^{-1}) = 1.695*VO_2 - 1.701*VCO_2 - 1.77*n$$

3.4.4 Exercise Protocols

3.4.4.1 Graded Exercise Test (VO_{2peak})

To ascertain the fitness level of participants, peak oxygen consumption (VO_{2peak}) was determined using a standard graded exercise protocol on an Excalibur Sport Lode Cycle Ergometer (Lode, Groningen, Netherlands). This involves 3 × 3 min submaximal workloads (*Males*, 50, 100 and 150 watts (W); *Females*, 25, 50 and 75 W) followed by successive 1 min workload increments of 25 W until volitional exhaustion. Respiratory gases were measured (section 3.4.3). Participants were encouraged to maintain a pedal frequency between 80 - 100 rpm and the test was terminated when this could not be maintained for a period of more than 5 s. Heart rate was monitored via telemetry (Polar Electro Inc., Oulu, Finland). VO_2 was considered maximum if a plateau was achieved (VO_2 increase of < 150 $ml.min^{-1}$ with increased workload). In the absence of a clear plateau, tests were still considered maximal efforts if at least two of the following secondary criteria were met: respiratory exchange ratio (RER) ≥ 1.10, a rating of perceived exertion (RPE) > 18, and/or maximum heart rate within ± 10 beats of age-predicted maximum. If such criteria were not met, then the highest VO_2 reached was termed VO_{2peak}. As not all participants achieved the criteria for maximal oxygen consumption (VO_{2max}) results are reported as VO_{2peak}. This value was used to determine relative individual workloads required during subsequent cycling experimental exercise tests.

3.4.4.2 Experimental Exercise Tests

Participants arrived at the laboratory at approximately 8am following an overnight fast, had their height and weight measured, and were fitted for a heart rate monitor (Polar Electro Inc., Oulu, Finland). Experimental exercise tests were separated by at least one week for males, and by one month for females who were tested in the proliferative phase of their menstrual cycle. Exercise protocols were performed on an Excalibur Sports Lode Cycle Ergometer (Lode, Groningen, Netherlands) with participants encouraged to maintain a pedal frequency between 80 - 100 rpm. Borg Scale ratings of perceived exertion (RPE 6 - 20 scale) (Table 3.2) were recorded every 10 to 15 mins (Chapters 5 and 7 respectively) throughout the exercise bouts. Exercise was preceded by a rest period and followed by passive recovery in a supine position. Details of study specific experimental exercise testing protocols are described within the relevant chapter.

RATING	PERCEPTION OF EFFORT
6	
7	Very Very Light
8	
9	Very Light
10	
11	Fairly Light
12	
13	Somewhat Hard
14	
15	Hard
16	
17	Very Hard
18	
19	Very Very Hard
20	

Table 3.2 The Borg Scale ratings of perceived exertion (RPE 6 - 20 scale). Adapted from Borg 1998.

3.4.5 Muscle Biopsies

Skeletal muscle biopsies were collected from the vastus lateralis tissue under local anaesthesia (1% Xylocaine, McFarlane Medical Supplies, VIC, Australia) by a trained physician. Three incisions were made in the skin at the site of the biopsy via a scalpel blade prior to conducting the exercise protocols described in Chapter 5 (HI and LO intensity exercise bouts) and no site was accessed more than once. Muscle samples were taken distal to proximal (at least 1 cm apart) in the middle of the muscle belly, approximately 5 - 8 cm above the kneecap. Muscle sampling was performed using the percutaneous needle technique (Bergström 1962) modified for suction (Evans et al. 1982) (Figure 3.2). Skeletal muscle samples were immediately snap frozen in LN_2 and ultra-freeze stored at -80ºC for later metabolomics analysis via gas chromatography mass spectrometry (GC-MS), as well as for the determination of m^6A methylation on RNA, and FTO mRNA and protein expression.

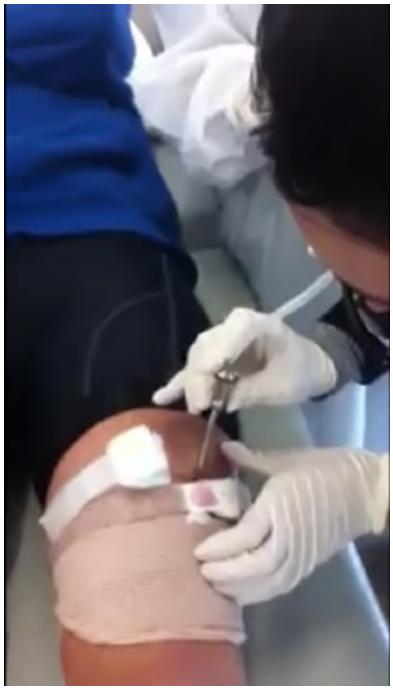


Figure 3.2 Skeletal muscle biopsy of the vastus lateralis performed under local anaesthesia by a training physician using the Bergström percutaneous needle technique modified for suction.

3.4.6 Metabolomics Analysis: Gas Chromatography Mass Spectroscopy (GC-MS)

3.4.6.1 Plasma Metabolite Extraction and Preparation

Plasma samples collected in Chapter 7 were removed from ultra-freeze storage (-80^{o}C) and permitted to slow-thaw on ice pending metabolite extraction. A total of 50 µl of each lithium heparin treated plasma sample was transferred into an eppendorf and diluted with 150 µl of methanol (MeOH) (spiked with 4% $^{13}C_6$-Sorbitol as an extraction internal standard (ISTD)). The samples were briefly vortexed and placed on ice for 10 mins before centrifugation at 13,200 rpm for 5 mins at 4°C to precipitate any protein present. A total of 100 µl of supernatant was then separated and transferred into 6 mm diameter conical bottom glass vial inserts (Phenomenex, NSW, Australia) that had been placed inside eppendorf tubes. An additional 10 µl of supernatant from each sample was combined into a separate eppendorf, which was vortexed and used to create 5 x 100 µl pooled biological quality control (PBQC) glass vial inserts. Eppendorf's containing the glass vial inserts were then dried *in vacuo* (RVC 2-33, John Morris, NSW, Australia) at a temperature of -55ºC and pressure of 3 mbar for 2-h. Glass vial inserts were then placed into 2 ml wide top glass vials and covered with metallic crimp caps (Agilent Technologies, CA, USA). Additional glass vials containing MeOX (methyloxime) (10 µl per sample) and TMS (trimethylsilyl) (20 µl per sample) were prepared for derivatisation.

3.4.6.2 Muscle Metabolite Extraction and Preparation

Muscle metabolite extraction, in preparation for GC-MS, was performed on skeletal muscle samples collected in Chapter 5. Muscle samples were cut in LN_2 to ensure that they remained frozen at all times. Approximately 20 mg wet weight of each skeletal muscle sample was added to a beaded cryotube with 250 µl of MeOH (spiked with 4% $^{13}C_6$-Sorbitol as an extraction ISTD). All samples were homogenised at -10°C using a cryomill in 3 x 30 s bursts at 6800 rpm interspersed by 45 s rest (Precellys 24, Bertin Technologies, Aix-en-Provence, France). The completely homogenised samples were then placed on a shaker at 850 rpm for 30 mins at 37°C prior to being centrifuged at 14,000 rpm for 10 mins. The supernatant was then separated and 100 µl of supernatant from each sample was transferred into 6 mm diameter conical bottom glass vial inserts (Phenomenex, NSW, Australia) that had been placed inside eppendorf tubes. An additional 10 µl of supernatant from each extracted sample was combined into a separate eppendorf, which was vortexed and used to create 8 x 100 µl PBQC glass vial inserts. Eppendorf's containing the glass vial inserts were then dried *in vacuo* (RVC 2-33, John Morris, NSW, Australia) at a temperature of -55°C and pressure of 3 mbar for 3-h. Glass vial inserts were then placed into 2 ml wide top glass vials and covered with metallic crimp caps (Agilent Technologies, CA, USA). Additional glass vials containing MeOX (10 µl per sample) and TMS (20 µl per sample) were prepared for derivatisation.

3.4.6.3 Instrumentation

The GC-MS system used comprised of a 7000B Agilent gas chromatograph triple-quadrupole and a 5975C Agilent triple-axis mass selective detector (Agilent Technologies, CA, USA) (Figure 3.3). A MPS2XL GC-MS autosampler (Gerstal Technologies, Mülheim, Germany) was set to select samples for analysis in a randomised order (Figure 3.3). MeOX and TMS derivatised samples were injected onto the GC column using a hot needle technique. The injection was operated in splitless (1 µl sample) and split (0.20 µl sample) modes to avoid overloaded chromatogram peaks. An inlet temperature of 230°C was set, and helium was used as the carrier gas with a flow rate of 1 $ml.min^{-1}$ along a 30 m VF-5MS column (with 0.25 nm film thickness) and a 10 m Integra guard column, for chromatographic separation (Varian Inc., VIC, Australia). The MS transfer line to the quadrupole was fixed at 280°C, the electron ionization (EI) ion source was fixed at 250°C, and the MS quadrupole was fixed at 150°C. The oven set up for sample analysis was: injection temperature of 70°C for a 1 min hold, followed by a $1°C.min^{-1}$ increase to 76°C (with no hold), and a $7°C.min^{-1}$ increase to a final temperature of 325°C for a 5 min hold. The GC-MS system was equilibrated for 1 min at 70°C prior to the injection of each sample. Ions were generated by a 70 eV electron beam at an ionisation current of 2.0 mA and spectra were recorded at 2.91 scans per second with a mass-to-charge ratio of 50 to 550 atomic mass units (amu) scanning range.

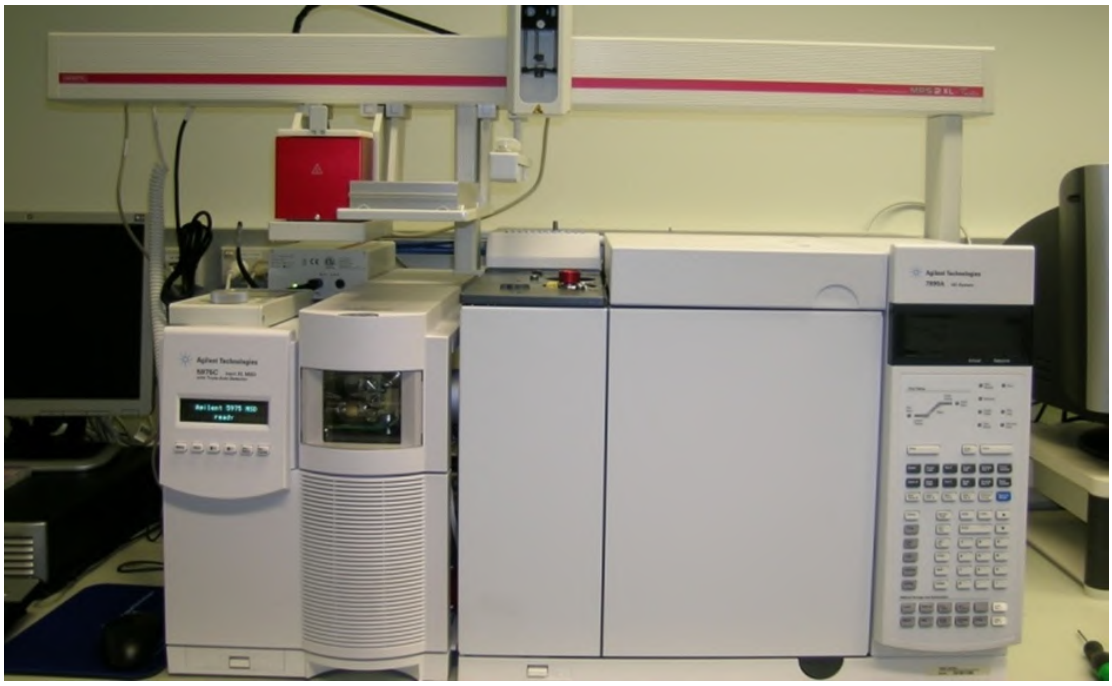


Figure 3.3 The 7000B Agilent gas chromatograph triple-quadrupole and 5975C Agilent triple-axis mass selective detector (Agilent Technologies, CA, USA). The MPS2XL GC-MS auto-sampler was used for randomising sample selection (Gerstal Technologies, Mülheim, Germany).

3.4.6.4 Data Handling

An Automated Mass Spectral Deconvolution and Identification System (AMDIS) was used to analyse the GC-MS chromatogram. A random PBQC was used for data filtering (to remove background noise), deconvolution (to align peaks and correct for retention time drifts between runs) and peak detection (via characteristics of each ion including *m/z* ratio, retention time and ion intensity). The mass spectra were further verified by analysis of reference standard compounds using the in-house Metabolomics Australia (University of Melbourne, Australia) mass spectra library. MassHunter was then used to analyse the relative response ratio (RRR) for each detected peak (the area of each unique ion) from the randomly selected PBQC, prior to this setting being applied to the remainder of the batch. Each individual sample was then cross checked to ensure the correct peaks had been selected in this process, and that peaks matched across all samples (Figure 3.4).

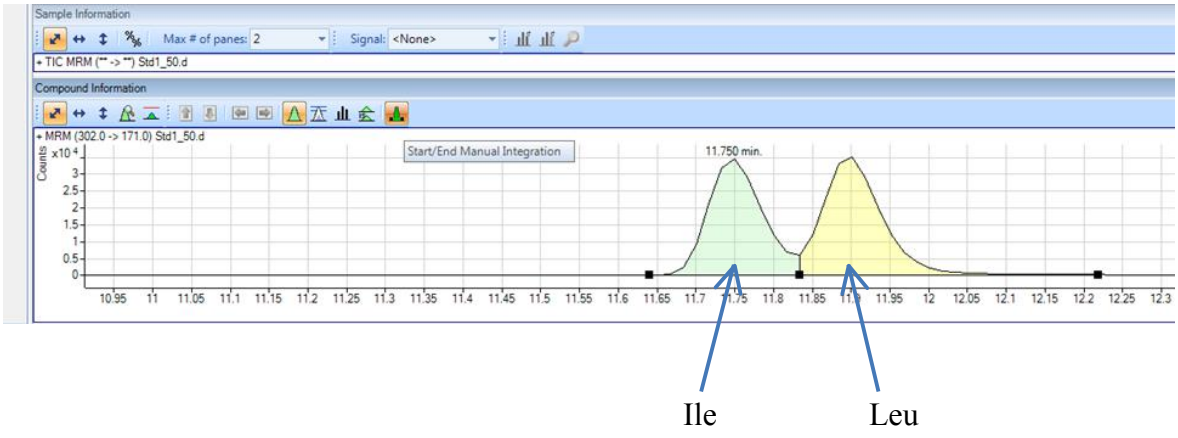


Figure 3.4 An example of ensuring correct peak picking using MassHunter. Leucine (Leu) elutes to the 10 m Integra guard column almost immediately following isoleucine (Ile). Each sample was cross checked to ensure the correct metabolite was selected for each peak based on the retention times set and peaks selected when initially analysing the random PBQC.

Metabolites were identified according to metabolomics standards initiative (MSI) level 1. Any overloaded peaks were analysed separately from the splitless mode. RRR data for each metabolite detected in each sample was normalised to the ISTD ($^{13}C_6$-Sorbitol). MetaboAnalyst 2.0 (Chapter 7) and 3.0 (Chapter 5) (Xia et al. 2012; Xia et al. 2015), in addition to SIMCA statistical modeling (version 14, MKS, Sweden), were used to generate multivariate pattern analysis models. Normalisation via a generalised log transformation was applied to each data matrix to improve symmetry prior to multivariate statistical analysis. Skeletal muscle samples from Chapter 5 were additionally normalised for wet weight.

Multiple terms are applied in metabolomics and at times these terminologies can be perplexing when used to define similar processes. Metabolite fingerprinting refers to "a snapshot of [the] intracellular metabolome" which can provide information on "metabolism at a single point of time" (Dunn et al. 2011). Metabolomics analysis of skeletal muscle is an example of this. Metabolic footprinting refers to the method of examining the "intracellular metabolism on the extracellular environment" (Dunn et al. 2011). An example of this is plasma, as this biological fluid can encompass metabolites secreted from a biological system (i.e. skeletal muscle) and therefore can be used to reflect changes in intracellular metabolism (Dunn et al. 2011). Throughout this dissertation the term 'metabolic signature' is used when referring to the investigation of patterns of correlated metabolites via multivariate statistical methods (Dunn et al. 2011). 'Metabolic profile' is used as a broad term to account for the univariate analysis performed in addition to multivariate statistical methods throughout this dissertation.

3.4.7 FTO Skeletal Muscle mRNA Expression

3.4.7.1 RNA Isolation, Reverse Transcription and cDNA Synthesis

Approximately 20 mg of frozen skeletal muscle tissue was cut in LN_2, weighed, and placed in a beaded cryotube. Samples were then homogenised using a Fast-Prep 24 (MP Biomedicals, NSW, Australia) in 500 µl of monophasic solution of phenol and guanidine isothiocyanate contained within TRIzol reagent. To prevent over heating of samples, the Fast-Prep 24 was set to speed setting 6 for 40 s, with samples rested on ice for 5 mins prior to a repeat homogenisation using the same conditions. To allow for complete dissociation of nucleoprotein complexes the muscle homogenate was left to sit for 5 mins at room temperature before being transferred from the beaded cryotube into an eppendorf. Following this, 100 µl of chloroform was added to the homogenate, and the solution was briefly vortexed and allowed to sit for a further 10 mins at room temperature before centrifugation at 12000 G for 15 mins at 4ºC. The supernatant was then removed and transferred into an eppendorf and 250 µl of isopropanol was added. This solution was again briefly vortexed, allowed to sit for 10 mins at room temperature, and centrifuged at 12000 G for 15 mins at 4ºC. The supernatant was then discarded and the RNA pellet washed with 500 µl of 75% ethanol and centrifuged at 7500 G for 5 mins at 4ºC. This washing step was repeated a second time before the supernatant was removed and the RNA pellet allowed to air dry for 10 mins prior to being resuspended in 30 µl of nuclease-free H_2O. Total RNA concentration was determined ($\mu g.\mu l^{-1}$) using a Nanodrop Spectrophotometer (Thermo Scientific, VIC, Australia), prior to samples being stored in an ultra-freezer at -80^{o}C until used for m^6A RNA methylation determination (section 3.4.9) and cDNA transcription (detailed below).

Total skeletal muscle RNA extracts were reverse transcribed to synthesize cDNA using an iScript cDNA Synthesis Kit (Bio-Rad Laboratories, VIC, Australia). The reverse transcription mixture consisted of 1 µg of the cellular RNA sample, 4 µl 5x iScript Reaction Mix, 1 µl iScript Reverse Transcriptase Buffer, and was made up to a total volume of 20 µl using nuclease-free H_2O. The mixture was incubated at 25^{o}C for 5 mins, 42ºC for 30 mins, and 85ºC for 5 mins. The resultant cDNA concentration ($ng.\mu l^{-1}$) was determined using a Nanodrop Spectrophotometer (Thermo Scientific, VIC, Australia) and samples were stored in an ultra-freezer at -80ºC until later analysis.

3.4.7.2 Assessment of FTO Gene Expression: Real-Time PCR Amplification

mRNA expression of FTO was assessed using a CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia). Oligonucleotide primers were designed according to known mRNA sequences of human skeletal muscle available online through the National Centre of Biotechnology Information (NCBI) database. Table 3.3 shows the details of the oligonucleotide primers (20X PrimePCR Assay's) that were synthesized commercially (Bio-Rad Laboratories, VIC, Australia). ß-Actin (ACTB) was used as an external control standard for each reaction due to it being considered as a constitutively expressed "housekeeping gene" and an appropriate external reference in RT-PCR in human skeletal muscle following acute exercise (Willoughby et al. 2007).

Primer Name	NCBI Reference Sequence	Amplicon Context Sequence	Amplicon Length (bp)
ACTB	NC_000007	GTGCTCGATGGGGTACTTCAGGGTGAGGATG CCTCTCTTGCTCTGGGCCTCGTCGCCCACATA GGAATCCTTCTGACCCATGCCCACCATCA	62
FTO	NC_000016.9	TTTTCTCCTCCAACGTTGTCATGGGCTTAAGC AAGAGCAGTGGAGACTTCTCTTGGCCCCTAG ATTGTAGCACCCGGGTCCCAATCCAAAACAG CTAGGAAATGGTGCCCATGAAGTTTTAAAT	94

Table 3.3 mRNA sequences of oligonucleotide primers used for the RT-PCR procedure as per the PrimePCR assay validation report (Bio-Rad Laboratories, VIC, Australia).

RT-PCR was conducted using a 96 well plate set up. Each reaction contained 100 ng of cDNA, 1 µl of 20X PrimePCR Assay and 10 µl SsoAdvanced Universal SYBR Green Supermix (Bio-Rad Laboratories, VIC, Australia). Nuclease-free H_2O was used to bring the total volume of each well to 20 µl. Following a 2 min activation phase at 95ºC, each sample underwent 40 amplifications on a CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia) (Figure 3.5) with each cycle consisting of 5 s of denaturation at 95ºC and 30 s annealing at 60ºC. At the end of 40 amplification cycles a melt curve involving 5 s/step at 65 - 95ºC (increasing in 0.5ºC increments) was implemented to confirm single gene products. The specificity of the PCR was demonstrated with absolute negative controls containing no cDNA.

Figure 3.5 The CFX96 Real-Time thermal cycler used for FTO and ß-Actin gene expression analysis (Bio-Rad Laboratories, VIC, Australia).

Fluorescence was measured after each cycle, resulting from the incorporation of SYBR green dye into the amplified PCR product. The relative expression of mRNA was assessed by determining the ratio between the cycle threshold (C_T) values of each target mRNA and the C_T for ß-Actin for each muscle sample obtained throughout the experimental exercise trials. The fold change in expression was then calculated using the fold change $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

3.4.8 FTO Skeletal Muscle Protein Expression

Skeletal muscle samples were prepared for protein expression determination by using a cryostat (Leica CM1950, Leica Biosystems, NSW, Australia) to shave 25 x 30 µm sections of each sample for analysis. During this process the cryostat chamber was set to -20ºC. Once the sections of each sample had been cut, they were carefully swept into a pre-frozen eppendorf that had been dunked momentarily into LN_2 prior to section collection. The skeletal muscle sections were then immediately placed into LN_2 prior to being stored at -80ºC for later homogenisation.

A total of 400 µl of homogenisation buffer [0.125 M Tris HCl, 10% Glycerol, 4% SDS, 10 mM EGTA, dissolved in pH 8.0] with DTT added fresh on the day (0.0154 g per ml being used) was added to each eppendorf containing skeletal muscle sample sections. These samples were permitted to sit at room temperature for 1-h or until completely homogenised, then were refrozen and stored at -80ºC prior to measuring protein determination.

3.4.8.1 Protein Determination

The protein concentration of each sample was determined colorimetrically using a RED 660 Protein Assay (G Bioscience, MO, USA). Homogenised samples were removed from ultra-freeze storage (-80ºC) and permitted to slow-thaw on ice pending analysis. A series of protein standards were prepared from a BSA stock solution [2 $mg.ml^{-1}$] diluted with homogenisation buffer to produce a final protein concentration range of 50 – 2000 $\mu g.ml^{-1}$.

On the day of each analysis 0.5 g of Neutralizer (G Bioscience, MO, USA) for every 10 ml of the RED 660 Protein Assay reagent was added to the reagent and vortexed until completely dissolved. This step was completed to account for the > 0.01% SDS used in the homogenisation buffer. Blanks, standards and diluted samples (10 µl) were added in triplicate to appropriate wells of a 96 well plate followed by 400 µl of reagent (with Neutralizer added). Plates were lightly tapped to mix and remove bubbles, and sat at room temperature for 5 mins. The proportion of protein in each well was determined spectrophotometrically at 660 nm using an X-mark microplate reader (Bio-Rad Laboratories, VIC, Australia). A standard curve was produced using the mean reading for protein standards minus the mean reading of the blank to determine the protein concentration (in $\mu g.\mu l^{-1}$) of each sample.

3.4.8.2 Western Blotting

Following protein determination homogenised muscle samples were removed from ultra-freeze storage (-80ºC) and permitted to slow-thaw on ice prior to pipetting the respective amount representative of 20 µg of total protein into a new eppendorf. In a separate eppendorf 90 µl of 4X Laemmli Sample Buffer (Bio-Rad Laboratories, VIC, Australia) was reduced by adding 10 µl of 2-mercaptoethanol for each participant's sample set. This mixture was then briefly vortexed and used to dilute each sample using a 3:1 sample to buffer ratio.

A 12% Mini-PROTEAN TGX Stain-Free Gel (10 x 50 µl wells) was assembled in a Mini-PROTEAN Tetra Cell electrophoresis chamber (Bio-Rad Laboratories, VIC, Australia). 1X Tris/Glycine/SDS running buffer was prepared by adding 100 ml 10X Tris/Glycine/SDS running buffer in 900 ml deionised H_2O. The 1X Tris/Glycine/SDS

running buffer was added to the inner and outer chambers of this system, with a disposable syringe used to rinse each well with the buffer. Following this, 3 µl of Precision Plus WesternC Standard (Bio-Rad Laboratories, VIC, Australia) was added to well 1, with the appropriate volume of samples (six samples/one participant per gel) loaded into wells 3 – 8 (well 2 was intentionally kept empty). The gels were run at 180 volts, and at varying ampules and watts, until the dye front reached the reference line. Gel cassettes were carefully opened using a lever, and the gel was gently removed and transferred onto an EZ Imager Gel Doc tray (Bio-Rad Laboratories, VIC, Australia). The stain-free gel was then activated and imaged using the EZ Imager Gel Doc using Image Lab software (Bio-Rad Laboratories, VIC, Australia).

Immediately following gel imaging, the gel was transferred using the Trans-Blot Turbo Transfer System (Bio-Rad Laboratories, VIC, Australia) at a mixed molecular weight setting (for ~7 mins per transfer). The Transfer Turbo cassette was prepared as per Figure 3.6 using a Trans-Blot Turbo Transfer Pack (Bio-Rad Laboratories, VIC, Australia) containing 0.2 µm PVDF with the gel placed carefully between layers. A roller was used to gently remove any bubbles that may have been present between layers prior to the transfer commencing.

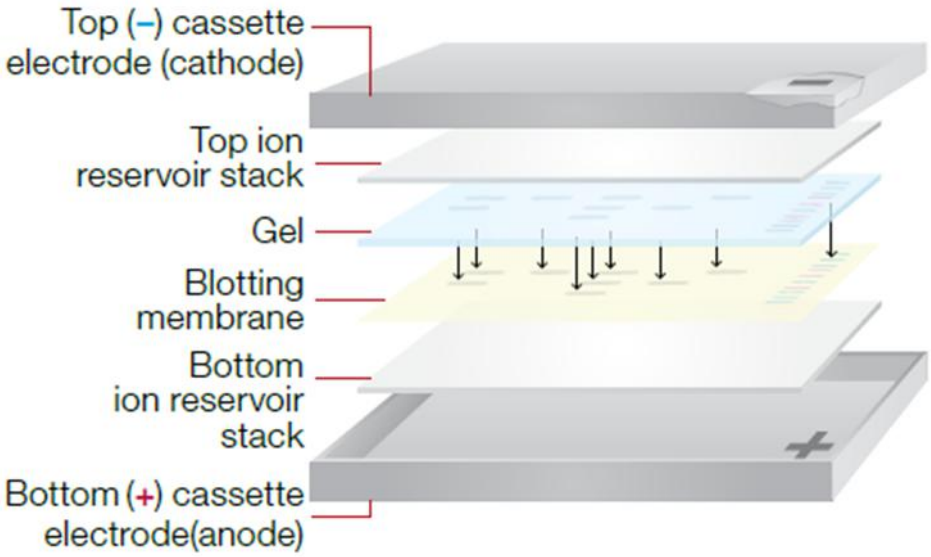


Figure 3.6 Trans-Blot Turbo Transfer set up for western blotting. Image sourced from Trans-Blot Turbo Blotting System Instruction Manual (Bio-Rad Laboratories, VIC, Australia).

Following the transfer process, the blotting membrane was immediately blocked with 5% skim milk powder in 1X TBST [50 mM Tris Base, 150 mM NaCl, pH 8.6 with 1M HCl, 0.5% Tween 20] for 1-h at 4ºC. Blocking solution was then removed from the membrane. A rabbit monoclonal primary antibody to FTO [EPR6894] (Cat. # GTX63821, GeneTex) was added to the membrane using a 1:1000 dilution within 5% skim milk powder in 1X TBST. The membrane was then placed on a rocker overnight at 4ºC. The standard ladder was treated separately with 0.5 µl Precision Protein StrepTactin HRP conjugate at a 1:10000 dilution within 5% skim milk powder in 1X TBST under the same conditions. The following morning these solutions were removed and the membrane was washed using 1X TBST for 4 x 15 min intervals on a rocker at room temperature, with wash solution removed and reapplied at each interval. Secondary antibody, goat anti-rabbit IgG H&L (HRP) (Cat. # ab97051, Abcam), was added to the sample membrane at a 1:5000 dilution within 5% skim

milk powder in 1X TBST, and placed on a rocker for 1-h at 4ºC. The wash step was then repeated.

Prior to detecting the FTO protein, the blotting membrane was added to Clarity Western ECL Substrate (Bio-Rad Laboratories, VIC, Australia) using a 1:1 ratio of peroxide reagent and luminol/enhancer solution, and allowed to sit in the dark at room temperature for 5 mins. The membrane was centred on a tray within a VersaDoc Imager (VersaDoc MP Imaging System, Bio-Rad Laboratories, VIC, Australia) and exposed chemiluminescently for 2 mins using the 'Chem Ultra-Sensitive' setting of Quantity One Software (Bio-Rad Laboratories, VIC, Australia) (Figure. 3.7). Immediately following the exposure process the blotting membrane was placed into 1X TBST to wash before re-commencing the blocking step previously detailed. The aforementioned steps were then repeated using the housekeeping protein mouse primary antibody pan Actin Ab-5 (ACTN05) (Cat. # MS-1295-P0, NeoMakers) at a 1:5000 dilution, and the secondary antibody anti-mouse IgG HRP (Cat. # NXA931, GE Healthcare) at a 1:10000 dilution.

The volume (intensity) of each FTO and Actin band was recorded using Image Lab Software (Bio-Rad Laboratories, VIC, Australia). FTO protein expression was calculated relative to the expression of Actin.

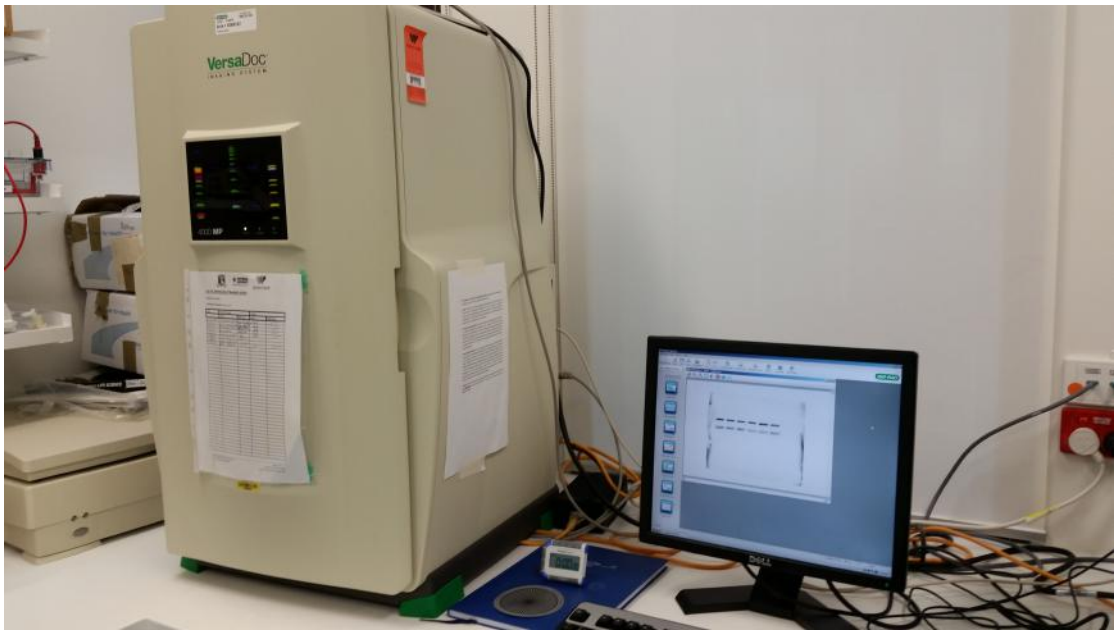


Figure 3.7 The VersaDoc Imager (VersaDoc MP Imaging System, Bio-Rad Laboratories, VIC, Australia) and Quantity One Software (Bio-Rad Laboratories, VIC, Australia) was used to image each membrane for the detection of FTO and actin bands.

3.4.9 m^6A RNA Methylation Determination

FTO protein function, via m^6A RNA methylation status of skeletal muscle samples, was measured colorimetrically using an EpiQuick m^6A RNA methylation Quantification Kit (Epigentek, Farmingdale, NY, USA). Isolated RNA samples (section 3.4.7.1) were permitted to thaw on ice whilst washing buffer was prepared by adding 26 ml of 10X wash buffer to 234 ml distilled water. To prepare standard curve solutions, 6 µl of a positive m^6A control [2 $\mu g.ml^{-1}$] was initially added to 18 µl of 1X TE buffer [10 mM Tris, 1 mM EDTA, pH 7.5 using HCl]. A series of m^6A standards were then prepared by further diluting the positive control to produce a final m^6A concentration range between 0.01 – 0.50 $ng.\mu l^{-1}$.

A total of 80 µl of binding solution was used to bind 2 µl of a negative control, each of the m^6A standards, and each RNA sample in duplicates to a strip well plate. For optimal quantification 200 ng of isolated RNA was used. Following 90 mins incubation at 37ºC the binding solution was removed from each well, prior to each well being washed three times using 150 µl of 1X washing buffer. The buffer solution was removed following each wash via pipette and the plate tap dried on absorbent paper. The m^6A on RNA was then captured immunospecifically by adding 50 µl of diluted capture antibody to each well (5 µl in 5 ml 1X wash buffer, 1:1000). The strip well plate sat covered at room temperature for 60 mins prior to each well being washed with 150 µl 1X wash buffer three times as described above. To detect m^6A, 50 µl of diluted detection antibody (2.5 µl in 5 ml 1X wash buffer, 1:2000) was added to each well. Following a 30 min incubation period at room temperature the wash step (as described above) was repeated four times. The detected signal was then enhanced to allow for high specificity to m^6A by adding 50 µl of diluted enhancer antibody (1

µl in 5 ml 1X wash buffer, 1:5000) to each well and allowing the strip well plate to incubate for a further 30 mins at room temperature. The wash step was then repeated five times.

The m^6A signal was detected by adding 100 µl of developer solution to each well, as this allowed for colour development. The strip well plate was then incubated in the dark at room temperature for 6 mins. In the presence of m^6A the colour of each well turned blue. At 5 mins following the addition of the developer solution, 100 µl of stop solution was added to each well to cease the aforementioned reaction, turning the combined solutions yellow. The amount of m^6A present in each well was quantified spectrophotometrically at 450 nm using an X-mark microplate reader (Bio-Rad Laboratories, VIC, Australia). A standard curve was produced using the mean readings of m^6A positive control standards minus the mean reading of the negative control (NC). Linear regression was conducted via Microsoft Excel software to determine the concentration of each unknown diluted sample using the follow formula:

$$m^6A\ (ng) = Sample\ (Ab_{450}) - NC\ (Ab_{450}) / Slope$$

3.5 Statistical Analysis

Results appearing in text, tables and figures are reported as mean ± standard error of the mean (SEM) unless stated otherwise. Diverse data handling and statistical techniques were employed from various software programs to perform univariate and multivariate statistical analysis throughout the different studies. As such, details of the statistical analysis performed in each study are described within the relevant chapter.

CHAPTER FOUR

A FTO gene variant and BMI comparison of resting metabolism and metabolic flexibility in males and females

4.1 Abstract

Metabolic flexibility refers to the ability of the muscle to effectively switch between substrates supplying energy based on availability. Although a decline in FTO transcription has been associated with alterations in glucose and lipid metabolism, it is unknown if this subsequently influences metabolic flexibility. This study investigated the influence of FTO rs9939609 genotype (variant alleles; AA, AT and TT) and BMI (lean; <25 kg/m^2 and overweight; >25 kg/m^2) on metabolic and respiratory markers of substrate oxidation in the basal state and following a glucose-stimulated dietary challenge. Apparently healthy, sedentary males and females aged 20 - 50 years (n = 147) completed a single experimental session designed to: 1) assess preprandial plasma glucose and serum insulin concentrations, RER and substrate oxidation following a 10 – 12 h overnight fast, and 2) assess the physiological responses of these variables following an oral glucose load (OGL) challenge. Metabolic flexibility (measured as ΔRER over time) was slower in overweight individuals compared to lean individuals (p = 0.009), but not different between FTO genotypes (p > 0.05), independent of BMI. Overweight individuals demonstrated lower preprandial fat oxidation (p = 0.012) and elevated preprandial glucose oxidation (p = 0.031) compared to lean individuals. No differences in preprandial or postprandial substrate oxidation were observed between FTO genotypes (p > 0.05). Plasma glucose was

significantly increased post OGL ($p < 0.001$), however, no differences were observed when separating participants by BMI or FTO genotype ($p > 0.05$). Significantly greater insulin resistance was observed in overweight individuals when compared to lean individuals ($p = 0.007$), whilst insulin resistance and sensitivity were similar between FTO genotypes ($p > 0.05$). An acute nutritional stimulus produced a slower metabolic flexibility and higher insulin resistance in individuals with an overweight BMI, but did not produce different responses between FTO allelic variants.

4.2 Introduction

Whole body energy balance requires a match in energy intake and energy expenditure, which ultimately equates to a balance between micronutrient intake and substrate oxidation at the tissue level (see review from Galgani & Ravussin 2008). Skeletal muscle is a large and highly metabolic tissue primarily involved in fat and glucose oxidation in the body. Metabolic flexibility describes the capacity of cellular metabolic pathways to adapt to substrate utilisation based on substrate availability (Aucouturier 2001; Storlien et al. 2004), and therefore can be used as a reflection of metabolic events occurring within the muscle. An impaired metabolic flexibility can exhibit as a diminished ability to oxidise fat in a basal state and transition to the use of carbohydrate in a prandial state driven by insulin (Kelly et al. 1999; Kelley et al. 1994). Metabolic inflexibility in the skeletal muscle has previously been related to the pathogenesis of insulin resistance (Kelley et al. 1999), and thus an impaired ability of the muscle to uptake glucose. However, the ability to alter substrate oxidation in response to nutritional stimuli may also depend on genetically determined factors (influencing cellular oxidation and substrate storage capacities) or epigenetic influences (Galgani et al. 2008*b*; Kulkarni et al. 2012).

Twin studies have shown that up to 70% of the variation to obesity susceptibility within a population is due to inter-individual genetic differences (Maes et al. 1997). Thus, genetic factors potentially predispose individuals to obesity in response to environmental perturbations (Hainer et al. 2000; Mustlein et al. 2009). The FTO gene has shown a robust association with obesity predisposition and a variety of obesity related traits, irrespective of age, gender and ethnicity (Jacobsson et al. 2012; León-Mimila et al. 2013; Mägi et al. 2013; Xi et al. 2013; Yako et al. 2015). Of interest is the FTO rs9939609 polymorphism, where individuals homozygous for the risk A-allele have a ~1.67-fold increased risk of becoming obese, compared to those who have not inherited the risk A-allele (TT genotypes) (Frayling et al. 2007).

Thus far, investigations have found no association between the FTO rs9939609 polymorphism and the ability to regulate substrate oxidation in a basal state or in response to nutritional stimuli in an obese cohort (Corpeleijn et al. 2010) and in a cohort comprising of elderly individuals (Grunnet et al. 2009*b*). However, both population subgroups have demonstrated impaired fat and glucose oxidation (Gumbiner et al. 1992; Kelley et al. 1999; Solomon et al. 2008) compared to their leaner and younger counterparts. Alternatively, an age-dependent decline in skeletal muscle FTO mRNA expression, rather than the FTO polymorphism, has been linked to peripheral defects in substrate metabolism in response to insulin infusion (via an EHIC), namely a lower oxidation of glucose and a higher oxidation of fat (Grunnet et al. 2009*b*). This implicates muscle and substrate utilisation in the metabolic mechanisms of FTO. In a different metabolic scenario, elevated FTO protein expression exists in T2DM patients (Bravard et al. 2011), who are characterised by

insulin resistance, and as such would expectedly exhibit a reduced stimulation of glucose oxidation and a blunted suppression of fat oxidation in insulin-stimulated conditions. Although this postulates that a similar metabolic effect may occur in the presence of opposing expression levels, this may be due to Grunnet et al. (2009*b*) examining associations with FTO mRNA and Bravard et al. (2012) examining associations with FTO protein. Grunnet et al. (2009*b*) did not examine whether an alteration in FTO protein expression or function occurred with the decline in FTO mRNA. Furthermore, alternate factors associated with aging (such as reduced muscle mass) could have contributed to the observed peripheral defects in substrate metabolism observed by Grunnet et al. (2009*b*). Notwithstanding these results, it is unknown if changes in FTO contribute to metabolic flexibility, and as such further exploration of whether FTO is associated with peripheral defects in the muscle is warranted. Nutritional stimuli, such as an OGL challenge, can perturb metabolic homeostasis and may assist in unmasking whether metabolic differences exist between allelic variants of FTO.

Therefore, the aim of this study was to examine if differences in FTO rs9939609 genotype, or BMI, existed in metabolic and respiratory markers of substrate oxidation following an OGL challenge. It was hypothesized that genotypes encompassing the risk A-allele and overweight individuals would display a slower metabolic flexibility compared the TT genotype and lean individuals in response to the OGL.

4.3 Methods

4.3.1 Participants

A total of 150 apparently healthy untrained males and females aged between 20 - 50 years were recruited to partake in this study, with participants excluded from participating if they met the exclusion criteria detailed in section 3.2. Those who met the eligibility criteria for this study were asked to provide written consent based on documents previously approved by the Victoria University Human Research Ethics Committee (*HRETH 12/197*) as per section 3.1. Participants were asked to refrain from consuming caffeine and alcohol, and from undertaking strenuous exercise for 24-h prior to attending the experimental trial. Participants recorded their dietary intake over 4 days prior to the trial, and were asked to consume a carbohydrate predominant meal (~60% of total macronutrient composition) the night before attending the laboratory. Experimental trials were conducted in the morning, 10 - 12 h after the last meal, to ensure participants were in a basal (preprandial) state.

4.3.2 Genotyping

The Taqman allelic discrimination assay (Life Technologies, VIC, Australia) was used for FTO rs9939609 (T>A) genotyping (see section 3.3). Fluorescence was visualised through a CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia). The overall genotyping efficiency was 98%, with undetermined results for 3 participants, resulting in a total data set of n = 147.

4.3.3 Anthropometric Measurements and Dietary Analysis

On the day of the experimental trial anthropometric and blood pressure measurements were recorded, and dietary records evaluated, as detailed in section 3.4.1. (see Supplementary Figure S-3.1 for the Diet and Activity Log template provided to participants).

4.3.4 Oral Glucose Load (OGL) Challenge

In order to examine glucose handling participants performed a standard OGL challenge which involved ingesting 75 g glucose in the form of a 300 ml solution (GlucoScan, SteriHealth, VIC, Australia). Participants were asked to consume the drink within a 2 min period for consistency (i.e. to standardise gastric emptying and intestinal absorption rates) between the subgroups.

4.3.5 Blood Sampling, Treatment and Analysis

A 20 ml venous blood sample was collected at 0 mins (pre OGL), and at 30, 60 and 120 mins (post OGL). Each blood sample was separated (as per section 3.4.2) and plasma was decanted and immediately analysed for glucose and lactate concentrations (see section 3.4.2.1). Serum samples of a subset population from each genotype group were decanted and analysed for insulin concentrations using an Insulin ELISA Kit (Invitrogen, VIC, Australia) as previously described in section 3.4.2.2. HOMA-IR and ISI were calculated using the indices listed in section 3.4.2.2. Plasma and serum samples were stored at -80^{o}C when not being used for analytical purposes.

4.3.6 Respiratory Gas Exchange Sampling and Analysis

Respiratory gas exchange sampling and analysis was conducted as previously detailed in section 3.4.3. Respiratory data were collected in 30 s intervals whilst lying down prior to and for 60 mins following glucose ingestion. Metabolic flexibility was calculated as the RER delta change (ΔRER) 60 mins post OGL compared to pre OGL measurements. This method has previously been used to determine metabolic flexibility in an insulin-stimulated state (Adamska et al. 2014; van de Weijer et al. 2013). Energy expenditure and substrate oxidation were calculated based on formulae described in section 3.4.3, with lean body mass (LBM) accounted for.

4.3.7 Statistical Analysis

Results are expressed as mean ± SEM. Statistical analysis was performed using SPSS software (IBM SPSS Statistics for Windows, Version 20, NY, USA). When necessary, raw data were log-transformed to obtain normality. Two-way ANOVA's with repeated measures were used to calculated individual significance in plasma, serum and indirect calorimetry data (energy expenditure, RER and substrate oxidation), with time as the within group factor and genotype or BMI as between group factors. Where an interaction was detected, multiple comparisons with Tukey's post hoc tests were completed to identify differences. One-way ANOVA's were performed for participant characteristic and nutritional intake data for genotype and BMI groups, with unpaired t-tests completed when interactions between factors were identified. Linear regression and covariant analysis (ANCOVA) were used to determine the effect of BMI and gender on allelic representation of dependent variables. The level of probability was set at $p < 0.05$.

4.4 Results

4.4.1 Characteristics of the Study Participants

Participant FTO genotype (rs9939609 variant alleles; AA, AT and TT) and BMI (lean; <25 kg/m^2 and overweight; >25 kg/m^2) groups are presented in Table 4.1.

Genotype Differences: Genotype frequency in the current study was consistent with Hardy-Weinberg equilibrium: 21.8% AA, 42.2% AT, and 36.0% TT. No difference in age, total body mass, height, BMI, hip and waist circumference, fat mass, muscle mass, water mass or blood pressure were observed when participants were separated by genotype for the FTO rs9939609 polymorphism ($p > 0.05$).

BMI Differences: Overweight participants displayed a higher total body mass, fat mass and muscle mass, as well as a larger hip and waist circumference, but lower total body water mass when compared to lean individuals ($p < 0.001$). Overweight and lean participants were similar in age and height and demonstrated similar blood pressure values ($p > 0.05$).

Clinical characteristics of the studied cohort based on their gender, with and without FTO genotype differentiation, are presented in Supplementary Tables S-4.1 and 4.2, respectively.

BMI	**< 25kg/m^2**	**> 25kg/m^2**		***P value***
n	87	60		
Age (yr)	28.3 ± 0.9	30.2 ± 1.0		0.178
Total Body Mass (kg)	63.5 ± 1.0	79.9 ± 1.6		**< 0.001**
Height (cm)	169.4 ± 1.0	168.8 ± 1.3		0.577
BMI (kg/m^2)	22.0 ± 0.2	28.1 ± 0.4		**< 0.001**
Hip Circumference (cm)	96.3 ± 0.6	108.2 ± 0.9		**< 0.001**
Waist Circumference (cm)	72.6 ± 0.8	84.0 ± 1.1		**< 0.001**
Fat Mass (%)	23.7 ± 0.8	31.8 ± 1.2		**< 0.001**
Muscle Mass (kg)	46.0 ± 0.9	51.9 ± 1.5		**< 0.001**
Water (%)	55.6 ± 0.5	49.6 ± 0.7		**< 0.001**
Systolic BP (mmHg)	122.1 ± 1.5	123.3 ± 1.7		0.613
Diastolic BP (mmHg)	73.6 ± 1.0	75.0 ± 1.1		0.338
Genotype	**AA**	**AT**	**TT**	
n	32	62	53	
Age (yr)	29.8 ± 1.7	28.8 ± 1.0	28.9 ± 1.2	0.839
Total Body Mass (kg)	70.7 ± 2.3	70.3 ± 1.5	69.9 ± 2.1	0.971
Height (cm)	169.0 ± 1.8	169.7 ± 1.2	168.3 ± 1.3	0.713
BMI (kg/m^2)	24.6 ± 0.6	24.5 ± 0.5	24.5 ± 0.6	0.981
Hip Circumference (cm)	102.1 ± 1.6	101.1 ± 0.9	100.9 ± 1.2	0.804
Waist Circumference (cm)	77.6 ± 1.7	77.0 ± 1.1	77.1 ± 1.4	0.958
Fat Mass (%)	27.5 ± 1.6	26.3 ± 1.2	27.4 ± 1.2	0.769
Muscle Mass (kg)	48.1 ± 1.8	49.0 ± 1.3	47.9 ± 1.5	0.830
Water (%)	52.7 ± 1.1	53.5 ± 0.8	53.0 ± 0.8	0.796
Systolic BP (mmHg)	121.9 ± 2.3	122.8 ± 1.1	122.8 ± 2.1	0.945
Diastolic BP (mmHg)	76.0 ± 1.6	72.7 ± 1.2	74.7 ± 1.2	0.190

Table 4.1 Clinical characteristics of the studied cohort based on their BMI and FTO rs9939609 genotype. Values are expressed as mean ± SEM. Significant differences (bolded, $p < 0.05$) were observed when grouping participants based on BMI.

4.4.2 Nutritional Intake Analysis

Genotype Differences: Daily caloric intake was similar between FTO genotypes ($p > 0.05$). A genotype interaction was detected for percentage carbohydrate intake ($p = 0.017$) and percentage saturated fat intake from total fat ($p = 0.020$), with subsequent analysis revealing that AA genotypes consumed a greater percentage of carbohydrates than AT genotypes ($p = 0.037$), and a larger percentage of saturated fat compared to TT genotypes ($p = 0.034$) (Table 4.2).

BMI Differences: No differences in average daily caloric intake, or percentage intake of carbohydrate, fat, protein, saturated fat, alcohol or fibre was observed between overweight and lean individuals ($p > 0.05$) (Table 4.2).

BMI	**< 25kg/m^2**	**> 25kg/m^2**		***P value***
Total Energy (kcal)	1794.2 ± 53.7	1870.0 ± 57.6		0.353
Protein (%)	21.4 ± 0.5	20.7 ± 0.6		0.626
Total Fat (%)	32.0 ± 0.7	33.7 ± 1.4		0.230
Saturated Fat (%)	40.3 ± 1.0	40.9 ± 0.8		0.668
Carbohydrate (%)	41.8 ± 0.8	41.8 ± 1.0		0.857
Alcohol (%)	1.5 ± 0.3	1.7 ± 0.4		0.793
Fibre (%)	2.4 ± 0.1	2.3 ± 0.1		0.768
Genotype	**AA**	**AT**	**TT**	
Total Energy (kcal)	1779.4 ± 79.7	1850.2 ± 63.0	1808.4 ± 61.2	0.768
Protein (%)	19.9 ± 0.9	22.1 ± 0.7	20.7 ± 0.6	0.089
Total Fat (%)	34.0 ± 2.4	31.8 ± 0.8	32.8 ± 0.9	0.460
Saturated Fat (%)	42.7 ± 1.4	40.2 ± 1.0	39.6 ± 1.1	**0.020**
Carbohydrate (%)	44.0 ± 1.3	40.9 ± 0.9	41.6 ± 1.0	**0.017**
Alcohol (%)	0.8 ± 0.3	1.8 ± 0.4	1.7 ± 0.4	0.237
Fibre (%)	2.4 ± 0.1	2.3 ± 0.1	2.4 ± 0.1	0.811

Table 4.2 Nutritional intake analysis of the studied cohort based on their BMI and FTO rs9939609 genotype. Values are expressed as mean ± SEM. Significant interactions (bolded, $p < 0.05$) were observed between FTO genotypes.

4.4.3 Substrate Utilisation and Metabolic Flexibility Analysis

A significant time effect for RER, energy expenditure (kJ.kgLBM^{-1}.min^{-1}), as well as glucose and fat oxidation (g.kgLBM^{-1}.min^{-1}) was observed post OGL ($p < 0.001$), regardless of genotype or BMI. Subsequent pairwise comparisons revealed a significant increase in RER, energy expenditure and glucose oxidation from pre OGL to 60 mins post OGL ($p < 0.01$). Conversely, fat oxidation was significantly reduced over the 60 min duration post OGL ($p < 0.01$).

Genotype Differences: Metabolic flexibility (ΔRER) was similar between FTO genotypes ($p = 0.299$) (Figure 4.1). The absence of an effect of BMI on metabolic flexibility response between FTO genotypes was confirmed by ANCOVA ($p = 0.267$, $R^2 = 0.020$). No genotype main effect, or genotype by time interaction was identified for RER, energy expenditure, or oxidation of fat and glucose ($p > 0.05$) (Table 4.3). When adjusted for BMI, energy expenditure and substrate oxidation also remained similar between FTO genotypes ($p > 0.05$) (Supplementary Table S-4.3).

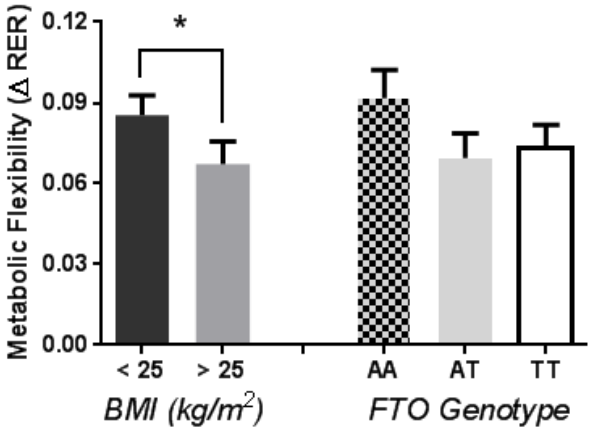


Figure 4.1 Metabolic flexibility measured as the ΔRER 60 mins post OGL compared to pre OGL measurements, with participants separated on their BMI and FTO rs9939609 genotype. Values expressed as mean ± SEM. * $p < 0.05$.

BMI Differences: Metabolic flexibility (ΔRER) was significantly slower in overweight individuals compared to lean individuals (p = 0.009) (Figure 4.1). No group main effect for RER was identified (p = 0.327), however, a group by time interaction was revealed (p = 0.010) (Table 4.3). Subsequent analysis revealed a significantly higher RER pre OGL in overweight individuals compared to their lean counterparts (p = 0.021), which indicates greater preprandial carbohydrate (in a basal state) in overweight individuals compared to lean individuals. This was confirmed by a group by time interaction identified for glucose oxidation (p = 0.004), with subsequent analysis revealing a significantly higher oxidation of glucose pre OGL in overweight individuals compared to their lean counterparts (p = 0.031). No group main effect for glucose oxidation (p = 0.349) was observed, however a weak trend for a group main effect for fat oxidation (p = 0.081) was apparent. A group by time interaction was identified for fat oxidation (p = 0.004), with subsequent analysis revealing a significantly lower oxidation of fat pre OGL in overweight individuals compared to their lean counterparts (p = 0.005). No group main effect (p = 0.237), or group by time interaction (p = 0.487) was identified for energy expenditure. The absence of an effect of gender on metabolic flexibility, energy expenditure and substrate oxidation responses between FTO genotypes was confirmed by ANCOVA (p > 0.05) (Supplementary Table S-4.3).

BMI	**< 25kg/m^2**		**> 25kg/m^2**				***P* value**
	Pre OGL	**Post OGL**	**Pre OGL**	**Post OGL**			
RER	0.80 ± 0.01	0.92 ± 0.01	0.82 ± 0.01	0.91 ± 0.01			**0.010**
EE (kJ.kgLBM^{-1}.min^{-1})	0.104 ± 0.002	0.118 ± 0.002	0.102 ± 0.001	0.113 ± 0.001			0.487
Glucose Oxidation (g.kgLBM^{-1}.min^{-1})	2.06 ± 0.16 x 10^{-3}	4.20 ± 0.12 x 10^{-3}	2.60 ± 0.18 x 10^{-3}	3.99 ± 0.15 x 10^{-3}			**0.004**
Fat Oxidation (g.kgLBM^{-1}.min^{-1})	1.76 ± 0.07 x 10^{-3}	1.18 ± 0.04 x 10^{-3}	1.48 ± 0.09 x 10^{-3}	1.17 ± 0.06 x 10^{-3}			**0.005**
Genotype	**AA**		**AT**		**TT**		
	Pre OGL	**Post OGL**	**Pre OGL**	**Post OGL**	**Pre OGL**	**Post OGL**	
RER	0.82 ± 0.01	0.93 ± 0.01	0.80 ± 0.02	0.92 ± 0.01	0.81 ± 0.01	0.92 ± 0.01	0.251
EE (kJ.kgLBM^{-1}.min^{-1})	0.102 ± 0.002	0.114 ± 0.003	0.104 ± 0.002	0.117 ± 0.002	0.104 ± 0.002	0.115 ± 0.002	0.679
Glucose Oxidation (g.kgLBM^{-1}.min^{-1})	2.47 ± 0.28 x 10^{-3}	4.08 ± 0.22 x 10^{-3}	2.16 ± 0.18 x 10^{-3}	4.16 ± 0.15 x 10^{-3}	2.31 ± 0.21 x 10^{-3}	4.13 ± 0.13 x 10^{-3}	0.630
Fat Oxidation (g.kgLBM^{-1}.min^{-1})	1.54 ± 0.10 x 10^{-3}	1.15 ± 0.06 x 10^{-3}	1.71 ± 0.08 x 10^{-3}	1.19 ± 0.06 x 10^{-3}	1.64 ± 0.10 x 10^{-3}	1.15 ± 0.05 x 10^{-3}	0.696

Table 4.3 RER, and calculated energy expenditure, glucose and fat oxidation pre OGL (0 mins) and 60 mins post OGL, with participants separated on their BMI and FTO rs9939609 genotype. Values are expressed as mean ± SEM. *P* value representative of group (genotype or BMI) by time interaction ($p < 0.05$, bolded). EE, energy expenditure; RER, respiratory exchange ratio.

4.4.4 Plasma Analysis

A significant main effect for time was observed for plasma glucose and plasma lactate concentrations following glucose ingestion ($p < 0.001$), regardless of genotype or BMI. Subsequent pairwise comparisons revealed a significant increase in plasma glucose from pre OGL to 30 mins ($p < 0.001$), 60 mins ($p < 0.001$) and 120 mins ($p < 0.05$) post OGL. Plasma lactate increased from pre OGL to 60 mins ($p < 0.001$) and 120 mins ($p < 0.001$) post OGL.

Genotype Differences: No genotype main effect (PG, $p = 0.114$; PL, $p = 0.407$), or genotype by time interaction (PG, $p = 0.427$; PL, $p = 0.307$) was identified for plasma glucose and plasma lactate concentrations (Figure 4.2 A & C).

BMI Differences: No group main effects were identified for plasma glucose and plasma lactate concentrations when separating participants by BMI (PG, $p = 0.203$; PL, $p = 0.301$). A group by time interaction was observed for plasma lactate ($p = 0.022$), with overweight individuals having lower plasma lactate concentrations at 120 mins post OGL (<25 kg/m^2, 1.55 ± 0.07 mmol.L^{-1}; >25 kg/m^2, 1.38 ± 0.03 mmol.L^{-1}; - 0.17 ± 0.08 mmol.L^{-1}; $p = 0.040$) compared to their lean counterparts (Figure 4.2 D). A moderate trend for a group by time interaction was observed for plasma glucose ($p = 0.064$) (Figure 4.2 B).

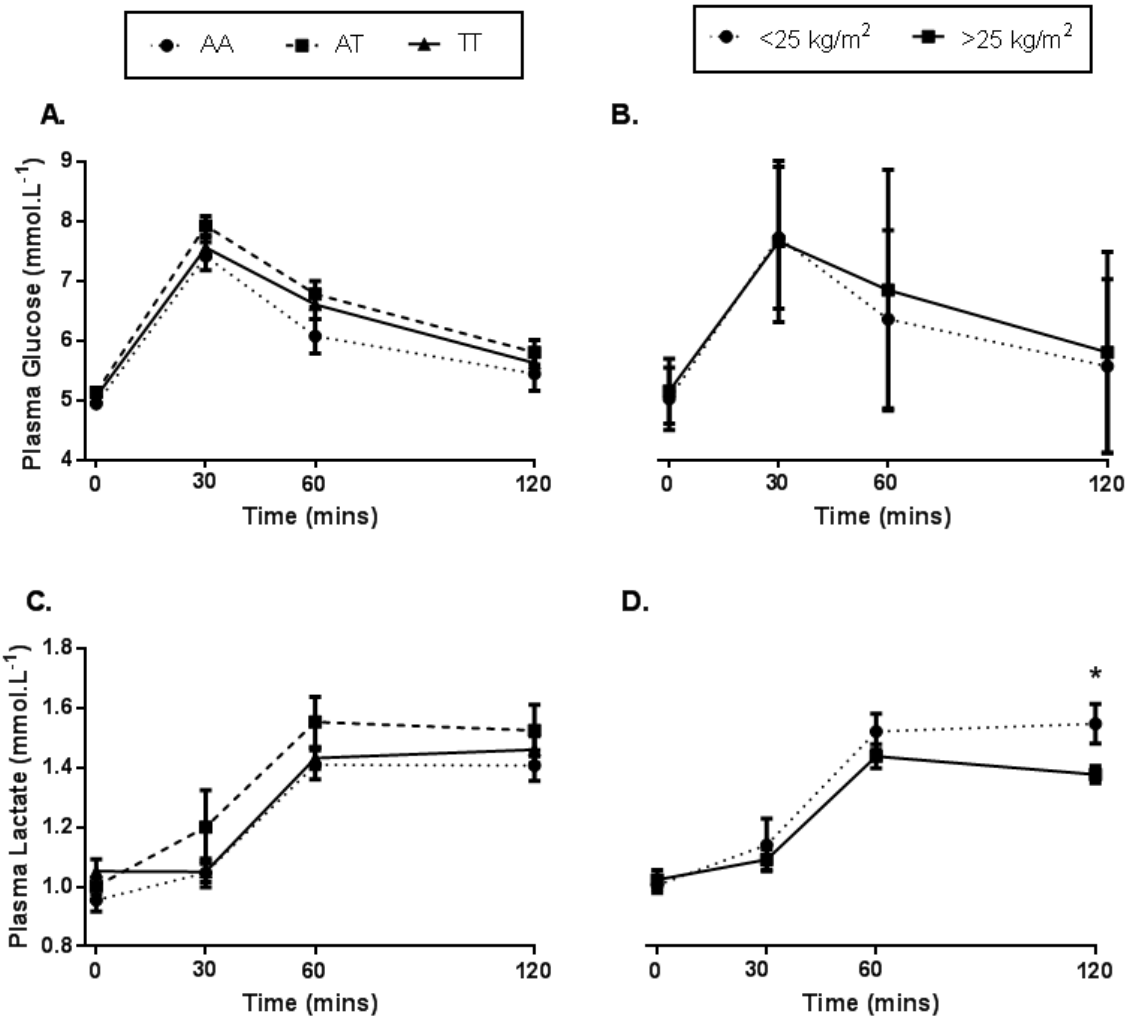


Figure 4.2 Plasma glucose and lactate concentrations observed prior to and following an OGL challenge between genotypes for the FTO rs9939609 polymorphism (A & C respectively) and BMI (B & D respectively). Values expressed as mean ± SEM. A main effect for time was observed in A to D in response to an OGL, $p < 0.001$. A group by time interaction was observed between BMI groups for plasma lactate at 120 mins (<25 kg/m^2 v. >25 kg/m^2), * $p < 0.05$.

4.4.5 Serum Analysis

Serum insulin concentrations were measured in a subset of 87 participants. Data obtained from this analysis was additionally used to examine BMI influences on this variable (n = 54 lean v. n = 33 overweight).

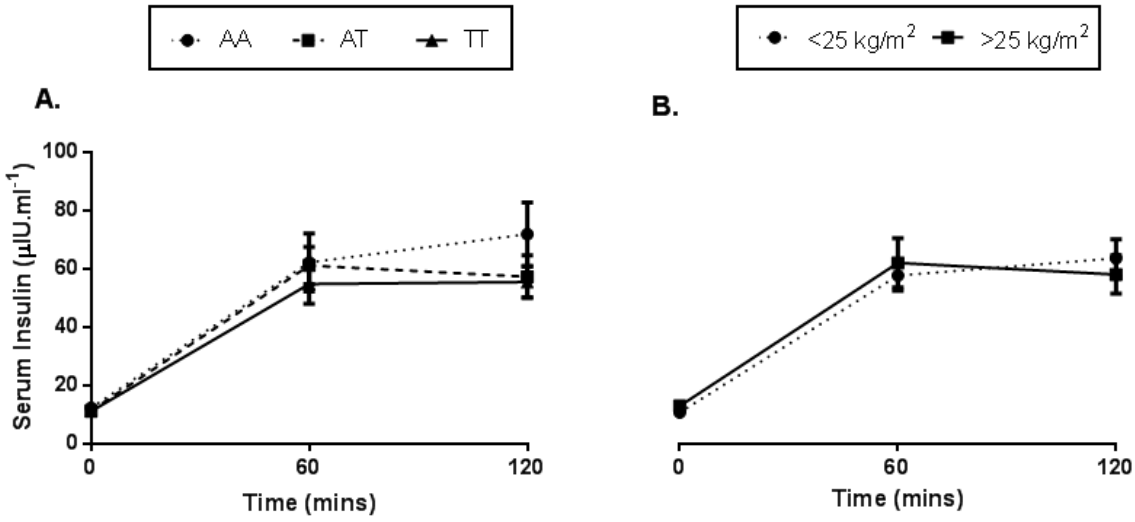


Figure 4.3 Serum insulin concentrations observed prior to and following an OGL challenge between genotypes for the FTO rs9939609 polymorphism (A) and BMI (B). Values expressed as mean ± SEM. A main effect for time was observed, regardless of BMI and genotype, in response to an OGL, $p < 0.001$.

A significant main effect for time was observed for serum insulin concentration post OGL ($p < 0.001$), regardless of genotype or BMI. Subsequent pairwise comparisons revealed a significant increase in plasma insulin from pre OGL to 60 mins ($p < 0.001$) and 120 mins ($p < 0.001$) post OGL.

Genotype Effects: No genotype main effect ($p = 0.439$), or genotype by time interaction ($p = 0.330$) was identified for serum insulin concentration when separating

participants by genotype (Figure 4.3 A). No difference in HOMA-IR (p = 0.382) or ISI (p = 0.828) was observed between genotypes (Figure 4.4 A & B).

BMI Differences: No group main effect (p = 0.951), or group by time interaction (p = 0.553) was identified for serum insulin concentration when separating participants by BMI (Figure 4.3 B). HOMA-IR was significantly higher in overweight individuals compared to their lean counterparts (<25 kg/m^2, 2.41 ± 0.78 v. >25 kg/m^2, 2.89 ± 0.77; + 0.48 ± 0.17; p = 0.007) (Figure 4.4 A). No difference in ISI was observed between BMI groups (p = 0.939) (Figure 4.4 B).

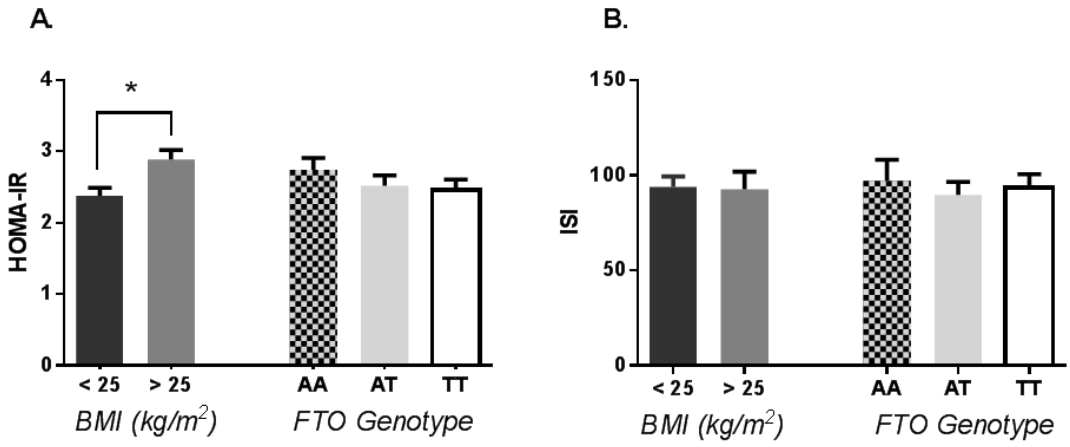


Figure 4.4 Insulin resistance (A) and sensitivity (B) measured using the HOMA-IR and composite ISI indices, respectively, with participants separated on their BMI and FTO rs9939609 genotype. Values expressed as mean ± SEM. * p < 0.05.

4.5 Discussion

The major finding of this study was that metabolic flexibility was similar between allelic homologues of the FTO rs9939609 polymorphism, independent of BMI and gender. The inability of the dietary challenge to reveal any metabolic differences between FTO genotypes suggests that, when nutritionally stimulated, a switch in substrate utilisation is similar in individuals carrying the FTO risk A-allele and the non-risk T-allele. However, when participants were separated by BMI, overweight individuals demonstrated a slower metabolic flexibility compared to lean individuals. Additionally, overweight individuals displayed lower basal fat oxidation, elevated basal glucose oxidation, and higher insulin resistance when compared to lean individuals. This supports previous reports of impaired metabolic flexibility to be associated with a greater adiposity and insulin resistance (Corpeleijn et al. 2008; Galgani et al. 2008a; Kelley et al. 1999).

The findings of the current study suggest that metabolic flexibility does not differ in individuals with FTO risk variants when compared to non-risk variants. In addition, rates of glucose and fat oxidation were similar across the three genotypes of the FTO rs9939609 polymorphism in a basal state and in response to insulin, which is supported by the metabolic flexibility data. In support of the current findings, another study has looked at the association between the FTO rs9939609 polymorphism and rates of fat and glucose oxidation in response to insulin-stimulation, and found no differences to exist between genotypes (Grunnet et al. 2009*b*). However, fundamental research using genetically engineered mouse models of eliminated (Fischer et al. 2009; Gao et al. 2010), reduced (Church et al. 2009) and enhanced (Church et al. 2010) *Fto* function provide support for a fundamental role of *Fto* in energy balance.

Global knockout of *Fto* results in reduced food intake, elevated energy expenditure, a loss of lean mass, and a reduced RER (likely representing protein catabolism) (Fischer et al. 2009), whilst a targeted hypothalamic knockout of *Fto* does not alter body composition, energy expenditure or RER but still decreases food intake (McMurray et al. 2013). Taken together, these findings indicate that a lack of *Fto* in the hypothalamus may explain only a small part of the phenotype observed in the global *Fto* knockout mice, indicating that *Fto* also promotes its biological effects through alternative, non-hypothalamic pathways. In a human cohort, levels of skeletal muscle FTO transcription have shown to be negatively associated to fat oxidation rates and positively associated to glucose oxidation rates in response to insulin-stimulation (Grunnet et al. 2009*b*), thus implicating muscle and substrate utilisation in the metabolic mechanisms of FTO. No other study has applied metabolic flexibility comparisons to genotypes of the FTO rs9939609 polymorphism, thus it is difficult to compare our findings to others. However, our results may signify that an acute nutritional stressor may not sufficiently influence changes at a cellular level. FTO was not measured in the current study and it is unknown whether differences in FTO protein level or activity exist in muscle between allelic variants. Furthermore, gender specific regulations of FTO on obesity risk have been indicated across paediatric, middle-aged and postmenopausal populations (Bouwman et al. 2014; Hubacek et al. 2009; Jacobsson et al. 2008; Saldaña-Alvarez et al. 2016). Although the current study examined a cohort that encompassed a higher proportion of females, the absence of metabolic flexibility, substrate oxidation and energy expenditure outcomes between FTO genotypes were independent of gender. Furthermore, the probability of type II error was found to be high when performing power analysis on RER (70.4%), carbohydrate oxidation (87.6%) and fat oxidation (89.3%) data (see supplementary

Figure S-4.4), indicating that a greater sample size may be required to detect potential dietary-induced changes to respiratory gases between FTO genotypes.

Estimated energy expenditure was similar across the three genotypes of the FTO rs9939609 polymorphism in a preprandial state and in response to the OGL. Previous investigations have also reported no relationship between energy expenditure and allelic variants of FTO genotypes in a basal state (Berentzen et al. 2008; Grunnet et al. 2009*b*; Speakman et al. 2008). Interestingly, Berentzen and colleagues (2008) initially found the AA genotype carriers to be associated with higher resting energy expenditure (in an age-adjusted model), however this association was eliminated when adjusting for lean body mass. Thus, elevated resting energy expenditure may be explained by a larger size of lean body mass accompanying increased fatness in the AA genotypes of FTO rs9939609 (Berentzen et al. 2008).

A slower metabolic flexibility was observed in overweight individuals in comparison to lean individuals, which indicates that the timeframe of the OGL was sufficient to detect metabolic flexibility differences. Previous investigations have reported a similar trend of metabolic inflexibility to be associated with a greater adiposity and insulin resistance (Corpeleijn et al. 2008; Galgani et al. 2008*a*; Kelley et al. 1999). Thus, observations of greater insulin resistance in overweight individuals compared to lean individuals were not unexpected. Along with elevated insulin resistance, lower preprandial fat oxidation in the overweight cohort compared to lean were not unexpected, as excess adiposity has also previously been linked with impaired fat oxidation in a basal state (Corpeleijn et al. 2008). Furthermore, in the current study preprandial glucose oxidation was greater in overweight individuals compared to their

lean counterparts. A blunted suppression of glucose oxidation, in addition to an impaired ability to oxidise fat whilst fasting, has previously been associated with metabolic inflexibility (Kelley 1999; Kelley et al. 1994; Kelley & Simoneau 1994). Overweight individuals also showed a significantly higher RER than lean participants prior to the OGL challenge, which reflects the elevated glucose oxidation observed for this group, and additionally, provides support for an impaired ability of those with higher adiposity to oxidise fat in a basal state (Corpeleijn et al. 2008). Metabolic inflexibility is typically associated with reduced glucose oxidation due to a reduced muscle uptake, and a blunted suppression of fat oxidation, when driven by insulin (Kelley 1999; Kelley et al. 1994; Kelley & Simoneau 1994). However, despite RER data in the current study being indicative of overweight individuals having a slower metabolic flexibility compared to their lean counterparts (as per previous discussion), similarities in postprandial fat oxidation were observed between BMI groups. Overweight individuals were found to have a 5% lower postprandial oxidation of glucose than their lean counterparts, which aligns with a typical metabolic inflexibility model, yet this was not statistically significant.

Plasma glucose concentrations were similar between FTO genotypes in this investigation. Similarities in plasma glucose concentrations between genotypes of the FTO rs9939609 polymorphism in response to various dietary challenges, including an OGL challenge, have also been observed by Grunnet et al. (2009*a*) and Wahl et al. (2014), when using substantially smaller sample cohorts (n = 46 and n = 56 respectively, compared to n = 147 in the current study). However, Grunnet et al. (2009*a*) did observe an association between the FTO risk A-allele in its homozygous form and elevated fasting blood glucose, compared with AT and TT genotypes. This

finding was not demonstrated in the current study, or by Wahl et al. (2014), and is yet to be replicated. This could be due to the deliberate inclusion of low birth weight adults by Grunnet and colleagues (2009*a*), as these individuals may also be predisposed to T2DM development (Barker et al. 1993), whilst the recruitment of this population subgroup was not an objective of Wahl et al. (2014) or the current study. Grunnet et al. (2009*a*) concluded that elevated hepatic insulin resistance is responsible for the elevated fasting blood glucose levels in AA genotypes, with no association between peripheral insulin resistance or insulin secretion and allelic variants of FTO detected. No genotypic difference for plasma glucose concentrations was reported by Kring et al. (2008) when using a larger cohort (n = 551) inclusive of obese and non-obese individuals, despite their investigation finding the AA genotype to be associated with decreased insulin sensitivity, compared to genotypes encompassing the non-risk T-allele. However, genotype associated differences in insulin sensitivity were concluded to have been mediated by the effect of total fat mass (Kring et al. 2008). The current study observed no difference between FTO allelic variants for insulin resistance or insulin sensitivity in response to the OGL challenge. However, when separated by BMI a significantly higher insulin resistance was observed in overweight individuals compared to their lean counterparts, which has been previously reported and linked to excess adiposity (Corpeleijn et al. 2008; Galgani et al. 2008*a*; Kelley et al. 1999).

Whilst FTO genotype did not influence plasma lactate response during the OGL challenge, BMI did, with overweight individuals exhibiting significantly lower plasma lactate concentrations at 120 minutes following glucose ingestion, when compared to their lean counterparts. Following glucose ingestion, plasma lactate

concentrations rise, representing a simultaneous stimulation of insulin stimulated glycolysis in the skeletal muscle (Berhane et al. 2015). Thus, a higher level of insulin resistance, evident in the overweight cohort, results in a reduced ability to uptake glucose in skeletal muscle which would subsequently reduce glycolysis and lactate production. A diminished capacity to produce lactate acutely in response to intravenous glucose exposure has been associated with progressively increased insulin resistance, more so than the degree of obesity per se (Lovejoy et al. 1992). More recently, this has been supported following an OGL challenge (Ho et al. 2013).

Current findings are similar to other studies that report caloric intake to not differ between genotypes of the FTO rs9939609 polymorphism (Bauer et al. 2009; Hakanen et al. 2009; Liu et al. 2010). However, previous reports have also indicated that risk variants of this gene enhance appetite and total energy intake (Speakman et al. 2008; Tanofsky-Kraff et al. 2009; Timpson et al. 2008; Wardle et al. 2008; Wardle et al. 2009). Whilst risk variants of FTO have also previously been associated with a greater preference for, and consumption of, energy dense foods (Brunkwell et al. 2013; Cecil et al. 2008; Sonestedt et al. 2009; Tanofsky-Kraff et al. 2009; Timpson et al. 2008; Wardle et al. 2009), dietary composition analysis found similarities in the total consumption of fat between genotypes. However, AA genotypes were found to consume greater amounts of saturated fat from total fat in comparison to TT genotypes. A high saturated fat intake can accentuate the obesity and metabolic syndrome risk associated with the FTO rs9939609 risk A-allele (Phillips et al. 2012). It is important to acknowledge the complexities of food intake and food preference, with many determinants influencing eating behaviours at both a household and population level (Thomas 1991). The validity and reliability of energy intake and food

composition variables may be limited due to the self-reporting nature of the data collection method, and could be further compounded by the relatively small cohort examined.

4.6 Concluding Remarks

This study confirmed that slower metabolic flexibility and altered substrate oxidation rates occurs in overweight individuals. No differences between allelic variants of the FTO rs9939609 polymorphism existed for metabolic flexibility or substrate oxidation. Additionally, although glucose ingestion exhibited the expected response of elevated plasma glucose and serum insulin concentrations, no difference across genotype alleles existed in the rate of elevation for these variables. Similar responses observed between genotypes suggest that allelic variants of FTO may not influence metabolic flexibility. However, it is possible that the metabolic stimulus produced by an OGL may not influence FTO function, or that the methodology selected to examine energy flux in this investigation (respiratory gas exchange analysis) wasn't sensitive enough to detect potential metabolic differences between genotypes. As such, it may be premature to conclusively state that FTO does not influence metabolic flexibility or specific metabolic pathways. More clarification may come from an adoption of more direct measurement of metabolic changes (i.e. the metabolomic analysis of skeletal muscle) in response to greater physiological stressors (i.e. acute exercise), and may offer more sensitive analysis to assist in unmasking the influence of the FTO on metabolic processes within peripheral tissues.

CHAPTER FIVE

Metabolic profiles of variants of the FTO rs9939609 polymorphism in response to high and low intensity exercise

5.1 Abstract

Under basal conditions and in response to acute nutritional stimuli, FTO has shown minimal impact on metabolism. Investigating exercise-induced changes in metabolism may lead to the identification of novel metabolic profiles and help identify any genetic influences from FTO on metabolism. The purpose of this study was to elicit exercise intensity dependent challenges on skeletal muscle metabolism, and to identify any specific skeletal muscle metabolic profiles that may exist between FTO genotypes. Twenty-eight apparently healthy untrained males and females were genotyped for the FTO rs9939609 (T>A) polymorphism, prior to performing isocaloric (400 kcal) cycle ergometer exercise on two separate occasions at different intensities: 80% (HI) and 40% (LO) VO_{2peak}. Skeletal muscle biopsies were sampled from the vastus lateralis pre exercise, and at 10 and 90 mins throughout passive recovery. GC-MS analysis of muscle samples identified 48 known metabolites. Changes were observed over the duration of the trials for 19 metabolites in the HI intensity exercise trial, and 13 metabolites in the LO intensity exercise trial ($p < 0.05$). However, multivariate models of metabolomic data (O2PLS-DA) were unable to detect any significant differences between genotypes with either exercise trial ($p > 0.05$). Univariate analysis of key responding VIP metabolites demonstrated greater skeletal muscle glucose accumulation at 10 mins following HI ($p = 0.021$) and LO (p

= 0.033) intensity exercise in AA genotypes compared to TT genotypes. No difference in plasma glucose and lactate concentrations, as well as glucose and fat utilisation, were observed between genotypes during each exercise trial ($p > 0.05$). A notable trend for a genotype by time interaction was observed for muscle fumarate/succinate ratio in response to the HI intensity exercise protocol ($p = 0.075$), with AA and AT genotypes having a greater accumulation, compared to the TT genotype, at 10 mins post exercise. Elevations in skeletal muscle glucose and fumarate/succinate following exercise in AA genotypes of the FTO rs9939609 polymorphism provide direction for future research on whether these observations are due to the function of FTO.

5.2 Introduction

The phenotypic characterisation of both Fto knockout and partial loss of function mice support the idea that the association of FTO SNPs with human obesity arises via the regulatory or functional influences of the FTO protein. Furthermore, the effects of *Fto* in the hypothalamus explains only some of the phenotype characteristic, especially in global *Fto* knockout mice, indicating that this protein promotes wider biological effects through other, non-hypothalamic influenced pathways. Under basal conditions, the association between genotypic variants of the FTO rs9939609 polymorphism and energy expenditure (Berentzen et al. 2008; Grunnet et al. 2009*a*; Speakman et al. 2008) or substrate oxidation (Grunnet et al. 2009*a*; Grunnet et al. 2009*b*) is not distinct. However, under conditions of stress (i.e. energy deprivation), FTO is impacted significantly leading to changes in energy homeostasis. Indeed, observations of reduced RER when *Fto* levels are manipulated in mice point to a role in peripheral metabolism and substrate utilisation regulation (Fischer et al. 2009; McMurray et al.

2013). In Chapter 4, an acute nutritional stress in the form of an OGL test was used to potentially influence FTO levels and/or function and thus unmask any differences in metabolic flexibility between risk and non-risk variants of FTO polymorphism. Despite no obvious differences between genotypes, utilisation of an exercise stress, which has opposing effects on muscle metabolism, may be another avenue for investigation.

Skeletal muscle is the main tissue involved in substrate utilisation. It is extremely adaptable to environmental changes, and its ability to expand energetic capacity and alter energy responses depending on requirements is central to substrate utilisation and energy balance (see review by Ferraro et al. 2014). In healthy individuals, an acute bout of exercise can alter the balance between glucose and fat oxidation rates in the skeletal muscle to a higher proportion of glucose utilisation with greater intensities (van Loon et al. 2001). Mouse models with a partial loss of *Fto* function (Fto^{I367F} mutation) have demonstrated altered substrate metabolism, namely a switch to a relatively greater contribution of carbohydrate metabolism than fat, as evident by elevated RER (Church et al. 2009). Conversely, a reduction in RER has been observed in adult onset *Fto* knockout mice, with the metabolic fuel switch reflecting an increased protein utilisation, most likely due to a greater loss of lean body mass, at the expense of carbohydrates (McMurray et al. 2013). The loss of lean body mass in knockout mice suggests that the FTO protein has an important role in the control of skeletal muscle. This may be due to an influence on nutrient and energy sensing, with the downregulation of FTO linked to glucose and essential amino acid deprivation (Cheung et al. 2013), decreased basal levels of mTORC1 signalling (Gulati et al. 2013), and elevations to TCA cycle intermediates fumarate and succinate (Gerken et

al. 2007). This could result in further consequences impacting a multitude of protein signalling cascades.

Phenotypic variability in BMI within FTO genotypes has been reported, highlighting the potential for environmental modifiers to influence the genetic burden of FTO risk variants (Yang et al. 2012). In particularly, the association between the risk A-allele of the FTO rs9939609 polymorphism and the odds of obesity can be attenuated by approximately 30% in physically active adults compared to those who are inactive (Kilpeläinen et al. 2011). Exercise can elevate the levels of metabolites that have known effects on skeletal muscle FTO protein such as fumarate and succinate (Gibala et al. 1997*a*; Gibala et al. 1997*b*) and offers a mechanism by which physical activity may attenuate FTO's influence on obesity predisposition.

The modifiable potential of exercise on FTO requires further investigation to determine the metabolic and cellular mechanisms involved. Since the investigation of metabolic interactions occurring in humans is complex, appropriate tools are required to detect and interpret the simultaneous interplay between genes and their ultimate influence on metabolism (Chorell et al. 2009). Metabolomics technology can offer a broad multiple metabolite analysis and specific metabolic responses to exercise may improve our understanding of genotype characteristics. Few studies have combined FTO genotyping with metabolomics characterisation, with plasma samples reflecting no unique metabolic signature in a basal state (Kjeldahl et al. 2014; Wahl et al. 2014) or in response to nutritional challenges (Wahl et al. 2014). Until now, no investigation has adopted a metabolomics approach to examine whether FTO genotype differences

in metabolic profiles exist in the skeletal muscle tissue, either pre exercise (in a basal state) or in response to exercise.

Therefore, the purpose of this project was to use two acute bouts of isocaloric exercise (at a high and low intensity) to elicit intensity dependent metabolic disturbances in the skeletal muscle and determine the potential of genotype influences on metabolic profiles in this tissue. This project also investigated whether differences between allelic variants existed for substrate utilisation during exercise. It was hypothesized that variations in metabolic profiles would exist between allelic homologues of FTO. It was additionally hypothesized that the ability to adjust substrate utilisation in response to metabolic demand would be reduced in risk A-allele carriers (AA and AT genotypes) compared to their homozygous non-risk allele counterparts (TT genotypes).

5.3 Methods

5.3.1 Participants

Twenty-eight apparently healthy untrained males and females provided written informed consent to participant in this study, which was approved by the Victoria University Human Research Ethics Committee (*HRETH 12/197*) and performed in accordance with the ethical standards set out in the 1964 Declaration of Helsinki. Participant exclusion criteria and pre experimental trial requirements are detailed in section 3.2. Participants were asked to record their dietary intake for 24-h prior to the first experimental exercise trial, and to replicate their intake in the 24-h prior to the subsequent trial.

5.3.2 Genotyping

Prior to the experimental exercise trials, cells from inside each participant's cheek were collected using a standard buccal swab, with QuickExtract solution (Illumina) used to extract DNA from these swabs. Genotyping of the rs9939609 (T>A) polymorphism of the FTO gene was performed using a Taqman allelic discrimination assay (Life Technologies, VIC, Australia) and a CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia) as described in section 3.3. The context sequence for the SNP tested was [VIC/FAM] GGTTCCTTGCGACTGCTGTGAATTT[A/T]GTGATGCACTTGGATAGTCTCTGTT. The overall genotyping efficiency was 100%.

5.3.3 Body Composition Assessment

Dual energy x-ray absorptiometry (DEXA; Hologic Discovery W, MA, USA) was used to assess body composition. Calibrations were performed the morning of DEXA analysis, and participants were in a standardised supine position throughout the duration of the scan. A whole-body scan was used (~1.5 mSv) to identify total body mass, fat mass, lean muscle mass and bone mineral content. Height, hip and waist circumference, and blood pressure were measured using a stadiometer, tape measure and sphygmomanometer (Omron HEM7322, Omron Healthcare, VIC, Australia) respectively.

5.3.4 Graded Exercise Test

To ascertain the fitness level of participants, VO_{2peak} was measured using the standard graded exercise protocol described in section 3.4.4.1. VO_{2peak} was determined approximately one week prior to the first experimental exercise trial. Data attained from the graded exercise test were used to calculate the workload each participant required for the subsequent experimental exercise trials at 80% and 40% of their VO_{2peak}.

5.3.5 Experimental Exercise Trial Protocol

Experimental exercise trials were conducted in the morning, approximately 10 - 12 h after the last meal to produce basal state conditions. Generic details regarding experimental exercise testing procedures are described in section 3.4.4.2. Participants were asked to complete a high (HI) and low (LO) intensity exercise protocol in a non-randomised order over the two experimental exercise trials: 1) **HI**, 80% VO_{2peak} (AA,

127.6 ± 13.1 W; AT, 126.0 ± 15.0 W; TT, 113.9 ± 15.1 W), and 2) **LO**, 40% VO_{2peak} (AA, 63.8 ± 6.6 W; AT, 62.9 ± 7.5 W; TT, 57.1 ± 7.6 W). Exercise was stopped once each participant had expended 400 kcal as estimated via indirect calorimetry. Energy expenditure and substrate utilisation were calculated based on formulae described in section 3.4.3. RER data was used to examine the response to metabolic demand by measuring the area under the curve (AUC) for RER transition from the beginning to the end of exercise. Borg Scale RPE data were recorded every 10 mins throughout the exercise bouts, and immediately upon cessation of exercise, to determine perceived physical demand between allelic variants of FTO (see Table 3.2 for the RPE scale used). Exercise was preceded by a pre exercise resting period and followed by 90 mins of passive recovery in a supine position.

5.3.6 Plasma Glucose and Lactate Analysis

Venous blood was collected from participants at each experimental exercise trial as per sampling procedures detailed in section 3.4.2. Blood were sampled pre exercise (0 mins), and at 10 and 90 mins throughout the post exercise passive recovery period. At each time point approximately 10 mls of venous blood was immediately placed into a lithium heparin tube (BD Vacutainer, BD Bioscience, NSW, Australia) and treated as per section 3.4.2. Plasma were decanted and analysed for plasma glucose and lactate concentrations (YSI 2300 STAT; Yellow Springs Instruments, Ohio, USA) (see section 3.4.2.1). Plasma albumin was measured using a commercially available BCG Albumin Assay Kit (see section 3.4.2.3) and used as an indirect marker and estimation of plasma volume changes during exercise (Maughan et al. 1985). Plasma samples were stored at -80°C when not being used for analytical purposes.

5.3.7 Muscle Biopsies

During each experimental exercise trial skeletal muscle biopsy samples were collected from the vastus lateralis tissue under local anaesthesia pre exercise (0 mins), and at 10 and 90 mins throughout the post exercise passive recovery period. Muscle biopsy preparation and collection procedures are described in section 3.4.5. Skeletal muscle samples were immediately snap-frozen in LN_2 and stored at -80°C until metabolomics analysis via GC-MS.

5.3.8 Metabolomics Analysis - GC-MS

5.3.8.1 Muscle Metabolite Extraction and Preparation

Approximately 20 mg of each skeletal muscle sample was used for metabolite extraction in preparation for GC-MS (see section 3.4.6.2 for full methodological detail). Each sample was spiked with 4% $^{13}C_6$-Sorbitol as an extraction internal standard (ISTD). Pooled biological quality control (PBQC) samples were created using 10 µl of supernatant from each extracted sample.

5.3.8.2 Instrumentation

The GC-MS system used (see Figure 3.3), and the settings of instrument conditions, for muscle metabolite analysis are described in section 3.4.6.3. During this study the GC-MS injection was operated in a splitless (1 µl sample) mode only (with no split mode) as pilot data had demonstrated no overloading of chromatogram peaks.

5.3.8.3 Data Handling

Data handling procedures, including mass spectra and peak verification procedures are detailed in section 3.4.6.4. Relative response ratio (RRR) data for each metabolite detected in each sample was normalised to the ISTD ($^{13}C_6$-Sorbitol) and to muscle sample wet weight. MetaboAnalyst 3.0 (Xia et al. 2015) was used to generate multivariate pattern analysis models, with normalisation via a generalised log transformation applied to the data matrix to improve symmetry prior to multivariate analysis. The Human Metabolome Database (HMDB) (Wishart et al. 2013) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000) database were used to create a visual metabolite network of the data via VANTED V2.1.0 (see Supplementary Figure S-5.1).

5.3.9 Data Analysis and Statistical Methods

Multivariate Analysis: An orthogonal two partial least squares discriminant analysis (O2PLS-DA) multivariate model was used as an analogous extension of the common PLS-DA model. This multivariate analysis model was selected due to its previously shown suitability in combining 'omics' data (Bylesjö et al. 2007). Model quality was reported for O2PLS-DA using $R^2X(cum)$ and Q^2, which represents, the measure of fit (i.e. the explained variation in metabolites) and the goodness of prediction (i.e. the variation in genotype that can be predicted by the model), respectively, as estimated by cross-validation (SIMCA statistical modeling, version 14, MKS, Sweden). The maximum possible Q^2 value is 1.0 as it is a fraction of the total variability, therefore $Q^2 \geq 0.7$ can be considered as a good predictor and < 0.5 as insignificant (Grootveld 2012; Wold et al. 1984). Likewise, the maximum possible $R^2X(cum)$ value is 1.0,

with this representative of a perfectly fitting model, whilst a $R^2X(cum)$ value of 0.0 would indicate no model fit at all (Eriksson et al. 2006). The AUC of the receiver operating characteristic (ROC) curve was used to determine the overall accuracy and separation performance of the genotypes in each O2PLS-DA model (Kumar & Indrayan 2011). PLS-DA was additionally performed on the whole set of metabolites (variables) at each time point to determine the metabolites with variable importance for projection (VIP) values ≥ 1.0 (Xia et al. 2015). The VIP value was used to reflect variable importance, and the metabolite subset with values ≥ 1.0 is herein referred to as 'VIP metabolites'.

Univariate Analysis: VIP metabolites were selected for further analysis (to reduce variability) and analysed using unpaired repeated-measures two-way ANOVA's. Where univariate analysis revealed any significant main effects for time, subsequent pairwise comparisons were performed to detect differences over time. Where a genotype by time interaction was detected, multiple comparisons with Tukey's post hoc tests were completed to identify differences. One-way ANOVA's were performed for participant characteristic and indirect calorimetry (substrate utilisation and RER AUC) data, with unpaired t-tests completed when interactions between factors were found. Linear regression and covariant analysis (ANCOVA) were used to determine the effect of age on allelic representation of dependent variables. Data are expressed as mean ± SEM unless otherwise stated. The level of probability was set at $p < 0.05$.

5.4 Results

5.4.1 Characteristics of the Study Participants

Similar participant characteristics were observed between FTO rs9939609 genotypes for total body mass, height, BMI, hip and waist circumference, fat mass, lean muscle mass, bone mineral content, blood pressure and VO_{2peak} ($p > 0.05$) (Table 5.1). Average workload cycled at during HI and LO intensity exercise trials were not different between allelic variants ($p > 0.05$). A genotype effect was detected for age ($p = 0.038$), with AT genotypes significantly older than TT genotypes ($p = 0.019$). The absence of an effect of age on respiratory gas exchange measurements and muscle metabolite responses between FTO genotypes was confirmed by ANCOVA ($p > 0.05$). A strong trend towards significance for a genotype effect was detected for fasting plasma glucose concentrations ($p = 0.057$).

	Genotype			*P value*
	AA	**AT**	**TT**	
n	10	9	9	
Gender	5F / 5M	4F / 5M	6F / 3M	
Age (yr)	24.4 ± 1.7	29.3 ± 2.2	22.7 ± 1.2	**0.038**
Total Body Mass (kg)	74.5 ± 3.9	72.0 ± 2.6	72.6 ± 4.2	0.875
Height (cm)	176.1 ± 2.8	169.9 ± 2.4	172.1 ± 1.9	0.212
BMI (kg/m^2)	24.0 ± 1.0	25.0 ± 1.1	24.4 ± 1.1	0.791
Hip Circumference (cm)	99.6 ± 2.4	99.6 ± 2.4	101.4 ± 1.8	0.813
Waist Circumference (cm)	81.8 ± 3.8	79.6 ± 2.8	77.8 ± 3.0	0.674
Fat Mass (%)	24.9 ± 1.6	22.6 ± 3.3	27.2 ± 2.0	0.416
Fat Mass (kg)	18.1 ± 1.0	16.1 ± 2.7	19.6 ± 2.0	0.486
Lean Muscle Mass (kg)	53.4 ± 3.5	52.3 ± 2.7	52.1 ± 3.3	0.689
Bone Mineral Content (kg)	2.6 ± 0.1	2.5 ± 0.1	2.5 ± 0.1	0.737
Systolic BP (mmHg)	129.0 ± 5.1	128.7 ± 4.2	122.7 ± 1.9	0.480
Diastolic BP (mmHg)	76.4 ± 3.2	75.8 ± 2.5	75.1 ± 3.0	0.952
Fasting Plasma Glucose ($mmol.L^{-1}$)	5.0 ± 0.1	5.4 ± 0.1	4.9 ± 0.1	0.057
VO_{2peak} ($ml.kg.min^{-1}$)	40.0 ± 1.4	39.0 ± 2.6	37.9 ± 2.3	0.771
80% VO_{2peak} Workload (W)	127.6 ± 13.1	126.0 ± 7.5	113.9 ± 7.6	0.767
40% VO_{2peak} Workload (W)	63.8 ± 6.6	62.9 ± 7.5	57.1 ± 7.6	0.779

Table 5.1 Participant characteristics when separated by FTO genotype of the rs9939609 polymorphism. Values are expressed as mean ± SEM. F, female; M, male.

Clinical characteristics of the studied cohort based on their gender, with and without FTO genotype differentiation, are presented in Supplementary Tables S-5.1 and 5.2, respectively.

5.4.2 Exercise Intensity and Physiological Markers

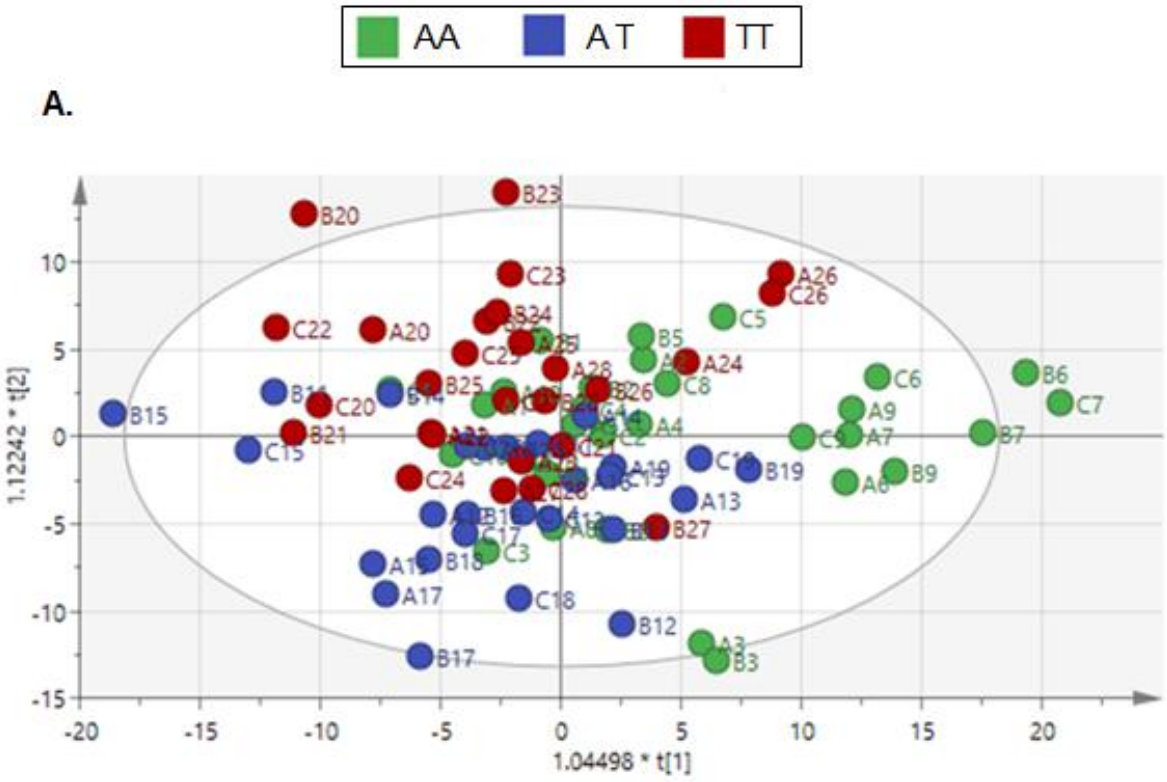
Both HI and LO intensity exercise trials elicited an increase in heart rate, with higher elevations during the HI trial compared to the LO trial (data not shown). Heart rate was similar between genotypes before, during and following HI and LO intensity exercise within each trial (data not shown). Additionally, RPE (considered on a numerical scale and presented as median (interquartile range)) was similar between genotypes at the completion of each exercise protocol (HI: AA, 16 (14 – 17), AT 16 (15 – 18), TT, 17 (15 – 19) (representing "Hard – Very Hard") (p = 0.254); LO: AA, 12 (11 – 13), AT 11 (11 – 12), TT, 13 (11 – 14) (representing "Fairly Light – Somewhat Hard") (p = 0.456)). There were no significant differences between genotypes in time to expend 400 kcal during the HI (p = 0.511) and LO intensity (p = 0.472) exercise protocol, or for average RER (HI, p = 0.323; LO, p = 0.603), glucose utilisation ($g.kgLBM^{-1}.T.I^{-1}$) (HI, p = 0.740; LO, p = 0.310) and fat utilisation ($g.kgLBM^{-1}.T.I^{-1}$) (HI, p = 0.709; LO, p = 0.498) measured during each exercise protocol (Table 5.2). The transition in substrate utilisation during exercise (RER AUC) was not significantly different between genotypes for each exercise protocol (HI, p = 0.206; LO, p = 0.410) (Table 5.2).

	Genotype			*P value*
	AA	**AT**	**TT**	
HI				
T.I (min:sec)	36:45 ± 2:00	39:29 ± 3:07	41:21 ± 3:02	0.511
Av. VO_2 (ml.kgbw.min^{-1})	29.0 ± 1.4	28.7 ± 2.1	27.0 ± 1.7	0.674
Av. RER	0.96 ± 0.07	0.99 ± 0.02	0.96 ± 0.01	0.323
RER AUC	62.7 ± 4.4	58.9 ± 5.8	71.9 ± 5.0	0.206
Fat utilisation (g.kgLBM^{-1}.T.I^{-1})	0.12 ± 0.04	0.10 ± 0.04	0.14 ± 0.03	0.709
Glucose utilisation (g.kgLBM^{-1}.T.I^{-1})	1.58 ± 0.13	1.68 ± 0.04	1.69 ± 0.12	0.740
Fat utilisation (%)	14.5 ± 4.5	10.2 ± 3.8	15.8 ± 3.2	0.593
Glucose utilisation (%)	85.5 ± 4.5	89.8 ± 3.8	84.2 ± 3.2	0.593
LO				
T.I (min:sec)	54:28 ± 2:58	57:59 ± 4:06	61:04 ± 4:19	0.472
Av. VO_2 (ml.kgbw.min^{-1})	20.0 ± 0.7	21.3 ± 1.2	19.0 ± 0.9	0.289
Av. RER	0.89 ± 0.01	0.91 ± 0.02	0.90 ± 0.01	0.603
RER AUC	82.6 ± 3.4	77.8 ± 6.5	86.4 ± 6.5	0.410
Fat utilisation (g.kgLBM^{-1}.T.I^{-1})	0.31 ± 0.04	0.24 ± 0.05	0.32 ± 0.05	0.498
Glucose utilisation (g.kgLBM^{-1}.T.I^{-1})	1.15 ± 0.08	1.33 ± 0.08	1.29 ± 0.10	0.310
Fat utilisation (%)	36.6 ± 4.0	30.1 ± 5.6	34.5 ± 4.3	0.607
Glucose utilisation (%)	56.4 ± 5.5	69.9 ± 5.6	57.2 ± 6.2	0.206

Table 5.2 Respiratory gas exchange measurements, and calculated fat and glucose utilisation, between FTO rs9939609 genotypes after isocaloric HI (80% VO_{2peak}) and LO (40% VO_{2peak}) intensity exercise. Values expressed as mean ± SEM. AUC, area under the curve; RER, respiratory exchange ratio; T.I, Time interval required to expend 400kcal.

5.4.3 Multivariate Analysis

Treatment of the chromatogram via AMDIS resulted in the detection of 48 identifiable metabolites (see Supplementary Table S-5.3 for metabolite identification details). Unpaired multivariate data models, O2PLS-DA with Pareto data scaling, were used to determine participant variation during the HI and LO intensity exercise trials, regardless of time (Figure 5.1). The O2PLS-DA modeling method demonstrated a similar metabolic signature between genotypes in the HI intensity exercise trial (p = 0.999), with very good validation metrics for data goodness of fit, $R^2X(cum)$ = 0.914, and very poor validation metrics for goodness of prediction, Q^2 = 0.084. Orthogonal variation in metabolites (X) accounted for 44% of the variation, and orthogonal variation between genotypes (Y) accounted for 32% of the variation. The O2PLS-DA modeling method also demonstrated a similar metabolic signature between genotypes in the LO intensity exercise trial (p = 0.982), with moderate validation metrics for data goodness of fit, $R^2X(cum)$ = 0.511, and very poor validation metrics for goodness of prediction, Q^2 = 0.017. Orthogonal variation in metabolites (X) accounted for 27% of the variation, and orthogonal variation between genotypes (Y) accounted for 17% of the variation.


AA
AT
TT
A.
1.12242 * t[2]
1.04498 * t[1]

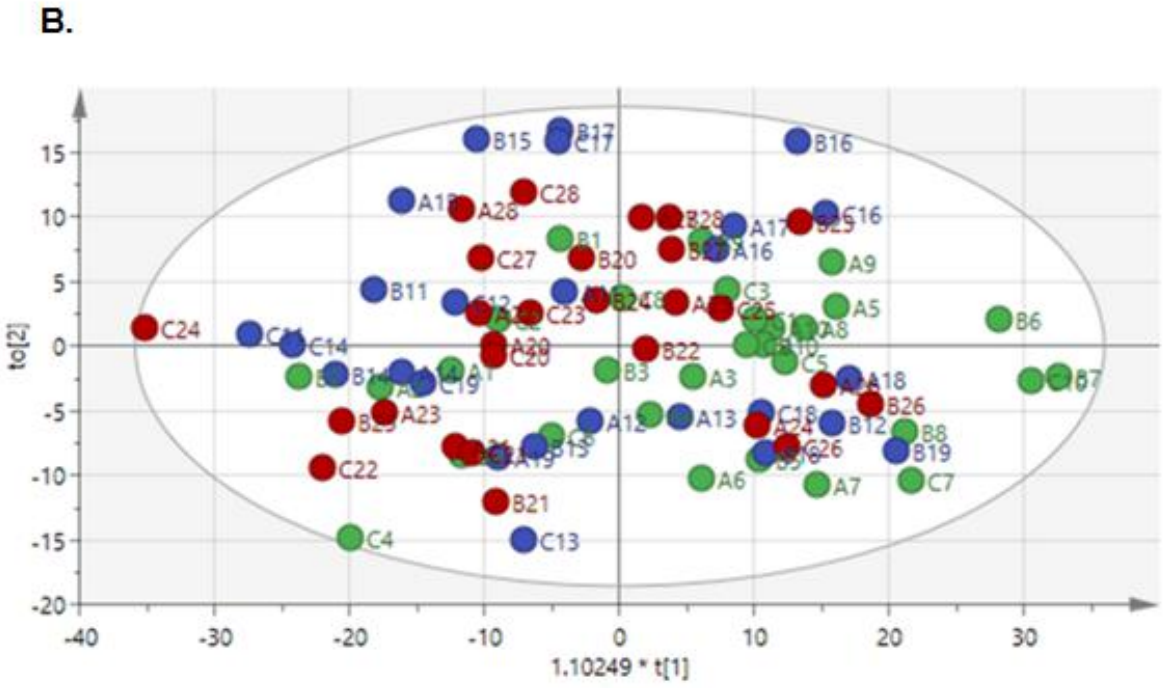

B.
to[2]
1.10249 * t[1]

Figure 5.1 O2PLS-DA models of genotype variation for the HI (A) and LO (B) intensity exercise trial. Green represent those homozygous for the risk A-allele (AA genotypes), blue is representative of heterozygous participants (AT genotypes), and red represents those who had not inherited the risk A-allele (TT genotypes). The ellipse is representative of a 95% confidence interval. Component 1 describes the orthogonal metabolite variation (within group variation) and Component 2 shows the primary variation between genotypes. Components are scaled proportionally to R^2X (A, $R^2X[1] = 0.099$, $R^2X[2] = 0.052$; B, $R^2X[1] = 0.346$, $R^2X[2] = 0.092$).

The metabolites associated with each genotype can be extracted from the loadings scatter plot (Figure 5.2). Distribution of metabolites in the direction of each genotype signifies their contribution to model variation due to the respective genotype, whilst metabolites with the least importance are clustered in the centre. The metabolites likely to contribute most to each genotype in the HI intensity exercise trial model were, AA: alanine, glutamate and glycine; AT: proline, adenosine monophosphate (AMP), urea and myoinositol; TT: glycerol-3-phosphate (glyercol-3-P), glycerate-3-phosphate (glycerate-3-P) and pyrophosphate. Data from the LO intensity exercise trial did not provide sufficient power to differentiate metabolite variation in relation to genotype using loading plot observations, or to generate a secondary predictive component. AUC of the ROC curve showed a poorer fit in the LO intensity exercise trial compared to HI intensity exercise trial, with the AA genotype better described by the model than the TT genotype (see Supplementary Figure S-5.2).

Correlation coefficient scores based on the weighted sum of the PLS regression were used to identify the top 10 metabolites with the greatest influence on the components at each time point, regardless of exercise intensity (Supplementary Figure S-5.3). PLS-DA cross validation determined 27 metabolites in total with VIP scores ≥ 1. These VIP metabolites were used for subsequent univariate analysis to determine metabolic changes over time and between genotypes for the HI and LO intensity exercise trials (see Supplementary Table S-5.4 for metabolites with VIP scores of ≥ 1 at each time point, regardless of intensity).

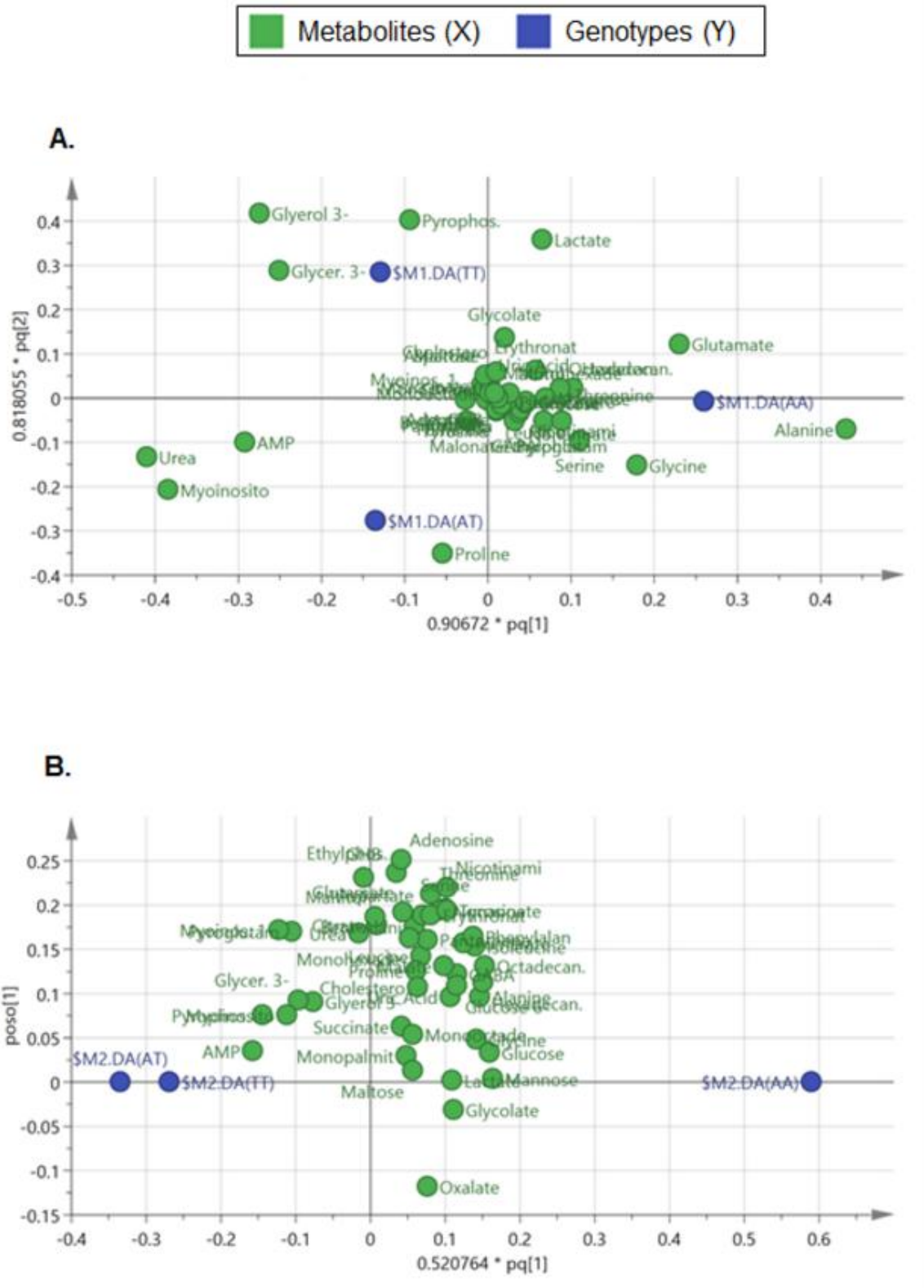


Figure 5.2 Loading plots of the O2PLS-DA models for the HI (A) and LO (B) intensity exercise trial. Genotype shown in blue and metabolites shown in green.

5.4.4 Univariate Analysis

5.4.4.1 The Effect of Exercise Intensity on Muscle Metabolites in Genotype Variants of FTO rs9939609

HI Intensity Exercise: A significant main effect for time was observed for skeletal muscle alanine, erythronate, fumarate, gamma hydroxybutyric acid (GHB), glucose, glutamate, glycine, glycolate, lactate, leucine, malate, maltose, mannose, monopalmitoglycerol, nicotinamide, phenylalanine, proline, tyrosine and uric acid following HI intensity exercise ($p < 0.05$) (Table 5.3). A strong trend for main effect for time was observed for muscle ß-alanine ($p = 0.052$) and glycerate-3-P ($p = 0.056$) following HI intensity exercise. At 10 mins post HI intensity exercise, muscle alanine, erythronate, fumarate, GHB, glucose, glycolate, lactate, malate, maltose, mannose, monopalmitoglycerol and tyrosine were significantly elevated compared to pre exercise ($p < 0.05$), whereas muscle glutamate and proline were significantly decreased ($p < 0.05$). A trend for lower muscle nicotinamide was detected at 10 mins post HI intensity exercise compared to pre exercise ($p = 0.065$). At 90 mins post HI intensity exercise, muscle erythronate and maltose were significantly elevated compared to pre exercise ($p < 0.05$), with a trend towards significance for higher levels for glucose ($p = 0.066$), glycolate ($p = 0.089$) and uric acid ($p = 0.060$). Conversely, muscle fumarate, glutamate, glycine, leucine, phenylalanine and proline were significantly lower at 90 mins post HI intensity exercise compared to pre exercise ($p < 0.05$). No main effect for genotype was identified for any of the VIP muscle metabolites ($p > 0.05$). A significant genotype by time interaction was observed for muscle glucose ($p = 0.036$), with subsequent analysis revealing a

significantly higher level of muscle glucose in AA genotypes compared to TT genotypes at 10 mins following HI intensity exercise ($p = 0.021$).

VIP Metabolite	HI			LO		
	Time	Genotype	Genotype x Time	Time	Genotype	Genotype x Time
Alanine	0.002			0.001		
ß-alanine						
Cholesterol						
Erythronate	0.004			0.008		
Ethylphosphate						
Fumarate	< 0.001			< 0.001		
GABA						
GHB	0.002					
Glucose	< 0.001		0.036	0.004		0.035
Glutamate	< 0.001			0.009		
Glycerate-3-P				0.004		
Glycine	0.010					
Glycolate	< 0.001			< 0.001		
Lactate	< 0.001			< 0.001		
Leucine	0.001					
Malate	< 0.001			< 0.001		
Maltitol						
Maltose	0.001			0.001		
Mannose	< 0.001					
Monopalmito.	0.032			0.034		
Nicotinamide	< 0.001					
Phenylalanine	< 0.001					
Proline	< 0.001					
Pyrophosphate				0.011		
Succinate						
Tyrosine	0.001			0.011		
Uric Acid	0.002					

Table 5.3 Skeletal muscle metabolites with a VIP value ≥ 1 in which a significant main effect for time, genotype main effect, or genotype by time interaction were determined. GHB, Gamma Hydroxybutyric Acid; GABA, Gamma Aminobutyric Acid; Monopalmito, Monopalmitoglycerol. $p < 0.05$.

LO Intensity Exercise: A significant main effect for time was observed for skeletal muscle alanine, erythronate, fumarate, glucose, glutamate, glycolate, glycerate-3-P, lactate, malate, maltose, monopalmitoglycerol, pyrophosphate and tyrosine following LO intensity exercise ($p < 0.05$) (Table 5.3). A trend for main effect for time was observed for muscle mannose ($p = 0.068$), uric acid ($p = 0.074$) and phenylalanine ($p = 0.086$). At 10 mins post LO intensity exercise, muscle alanine, erythronate, fumarate, glucose, glycolate, lactate, malate, monopalmitoglycerol and tyrosine were significantly elevated compared to pre exercise ($p < 0.05$), with a trend for elevated muscle maltose ($p = 0.060$). At 90 mins post LO intensity exercise, muscle erythronate, glutamate, glycerate-3-P, glycolate, lactate, monopalmitoglycerol and pyrophosphate were significantly elevated compared to pre exercise ($p < 0.05$), while only a trend towards significance for elevated fumarate ($p = 0.064$), maltose ($p = 0.068$) and glucose ($p = 0.074$) were observed compared to pre exercise. No main effect for genotype was identified for any of the VIP muscle metabolites ($p > 0.05$). Similar to the HI intensity exercise trial, a genotype by time interaction was observed for muscle glucose ($p = 0.035$), with subsequent analysis revealing a significantly higher level of muscle glucose in AA genotypes compared to AT ($p = 0.028$) and TT ($p = 0.033$) genotypes at 10 mins post LO intensity exercise.

5.4.4.2 Skeletal Muscle Fumarate/Succinate Analysis

A significant main effect for time was observed for skeletal muscle fumarate/succinate following both HI and LO intensity exercise ($p < 0.001$) (Figure 5.3 A & B). Subsequent pairwise comparisons revealed a significant increase in muscle fumarate/succinate from pre exercise to 10 mins ($p < 0.001$) in the HI intensity exercise trial, and a significant decrease in muscle fumarate/succinate from pre exercise to 90 mins ($p < 0.001$) in the LO intensity exercise trial.

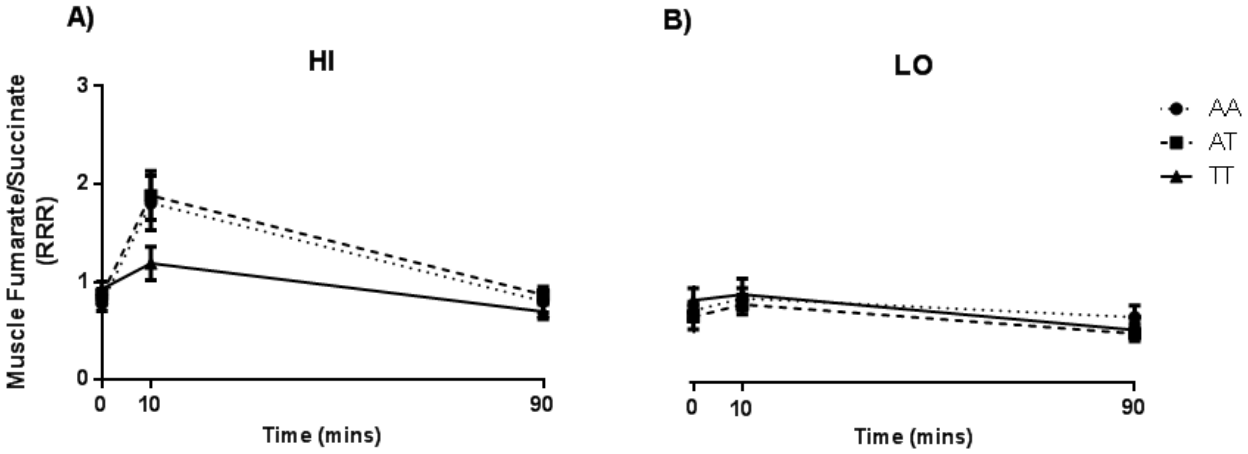


Figure 5.3 Effect of exercise intensity on fumarate to succinate ratio in the vastus lateralis skeletal muscle. Data is expressed as mean ± SEM of the Relative Response Ratio (RRR) of these metabolites to the ISTD ($^{13}C_6$-Sorbitol) throughout the A) 80% VO_{2peak} (HI) and B) 40% VO_{2peak} (LO) intensity exercise.

Whilst no genotype main effect ($p = 0.397$) was observed for muscle fumarate/succinate in response to HI intensity exercise, a trend towards significance for a genotype by time interaction ($p = 0.075$) was detected. No genotype main effect ($p = 0.693$), or genotype by time interaction ($p = 0.570$) was detected for muscle fumarate/succinate in response to LO intensity exercise.

5.4.5 The Effect of Exercise Intensity on Plasma Metabolites in Genotype Variants of FTO rs9939609

The current investigation used plasma albumin concentrations as an indirect marker of plasma volume changes in response to exercise (Maughan et al. 1985). Exercise-induced changes in plasma albumin concentrations were similar between genotypes at all observed time points during both exercise trials (data not shown) ($p > 0.05$).

A significant main effect for time for plasma glucose was observed in the HI intensity exercise trial ($p < 0.001$). Subsequent pairwise comparisons revealed significantly higher plasma glucose at 10 mins ($p = 0.001$) and 90 mins ($p = 0.042$) post HI intensity exercise compared to pre exercise (Figure 5.4 A). No main effect for time for plasma glucose was detected in the LO intensity exercise trial ($p = 0.533$) (Figure 5.4 B). No genotype main effect (HI, $p = 0.656$; LO, $p = 0.196$), or genotype by time interaction (HI, $p = 0.681$; LO, $p = 0.932$) was identified for either exercise trial.

A significant main effect for time was observed for plasma lactate concentrations during the HI and LO intensity exercise trials ($p < 0.001$). Subsequent pairwise comparisons revealed significantly higher plasma lactate at 10 mins ($p < 0.001$) compared to pre exercise for both the HI and LO intensity exercise trials, and at 90 mins ($p < 0.001$) compared to pre exercise for the HI intensity exercise trial (Figure 5.4 C & D). No genotype main effect (HI, $p = 0.471$; LO, $p = 0.358$), or genotype by time interaction (HI, $p = 0.333$; LO, $p = 0.145$) was identified for either exercise trial.

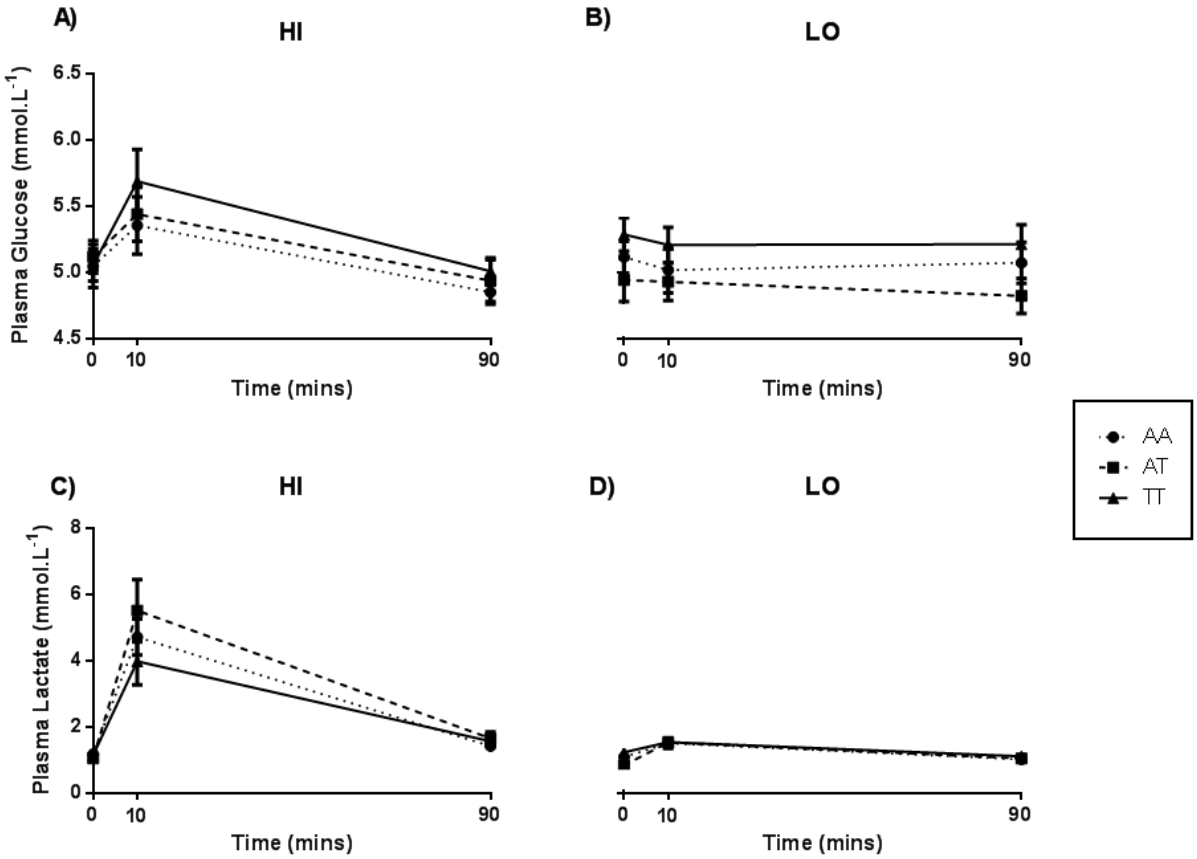


Figure 5.4 Plasma glucose and lactate concentrations between genotypes of the FTO rs9939609 polymorphism prior to and following (during passive recovery) isocalorically matched HI (A & C) and LO (B & D) intensity exercise. Values expressed as mean ± SEM.

5.5 Discussion

The current investigation used two acute bouts of isocaloric exercise at high and low intensity to determine whether genotype specific metabolic profiles of intramuscular metabolism existed. High intensity exercise elicited greater metabolic perturbations with 25 metabolites (out of the 48 detected), predominately of carbohydrate and protein metabolism, demonstrating changes occurring during and post exercise. Conversely, only 18 metabolites, most of which were related to carbohydrate metabolism, responded to the low intensity exercise protocol. Despite this, multivariate analysis revealed no distinct differences between genotypes of the FTO rs9939609 polymorphism in response to either exercise protocol. Notwithstanding, univariate analysis of VIP metabolites revealed elevated levels of muscle glucose during the early stages of the recovery period following both high and low intensity exercise in individuals homozygous for the risk A-allele compared to the non-risk T-allele. The findings potentially support previous research suggesting that FTO may influence carbohydrate metabolism, however, whether this has a more chronic effect and contributes to FTO's association with obesity and T2DM requires further investigation.

O2PLS-DA multivariate regression was used in the current study to model genomic and metabolomic data, and identify whether metabolic signature differences were present between allelic variants of FTO following high and low intensity exercise. Although this model was unable to identify a distinct metabolic separation for each allelic variant of FTO following both exercise trials, it was demonstrated that the higher intensity exercise showed greater metabolic perturbations, evidenced by an observed effect on 25 metabolites compared to 18 in the low intensity trial (see

Supplementary Table S-5.5). Previous research has shown acute bouts of high intensity exercise to perturb metabolism to a greater extent than exercise of low-moderate intensity (Egan et al. 2010; Peake et al. 2014), and thus the ability of high intensity exercise to stimulate greater metabolic changes in the current study was not unexpected. Throughout the data analysis phase of this study we were unable to generate a multivariate model that took changes at specific time points into account for individual genotypes, and to the author's knowledge no such model currently exists. Rather, the O2PLS-DA models of metabolic profiles during each exercise protocol combined pre exercise and post exercise samples. Incorporating a pre exercise time point may potentially confound observations, with previous metabolomics investigations having found similar metabolic profiles between allelic variants of FTO in a basal state (Kjeldahl et al. 2014; Wahl et al. 2014). However, these studies may be limited by only analysing plasma samples, which can be influenced by factors other than the skeletal muscle, such as hepatic output. It is also unknown whether the incorporation of a time point in which energy demand was at a minimum (pre exercise), and tight homeostatic regulation expected, may account for the absence of genotype specific metabolic signatures in the current study. Thus, the development of a multivariate model that enables differentiation of genomic-metabolomic data from specific time points may provide a greater separation between genotypes. Furthermore, when using O2PLS-DA, the standard deviation in metabolites was found to be large, resulting in a reduced confidence, and contributing to the lack of Q^2 reliability. The very poor Q^2 observed for both the high and low intensity exercise data may also suggest that the sample size was not large enough, given the total variation. Additionally, the high disparate (>25%) in $R^2X(cum)$ and Q^2 values indicates that the multivariate analysis model used may have faced an over-

fitting problem (Worley & Powers 2013), thus leading to a poor predicative performance. Over-fitting can occur with a large number of metabolites and small sample sizes (Xi et al. 2014), and as all 48 detected metabolites were used when completing the analysis (rather than only VIP metabolites per se) care should be taken when interpreting the current results.

Univariate analysis was performed on the 27 VIP metabolites following both acute bouts of exercise. A major finding from this analysis was that individuals homozygous for the risk A-allele demonstrated greater muscle glucose levels compared to individuals homozygous for the non-risk T-allele in the early stages of recovery following both high and low intensity exercise. Similar to TT genotypes, a lack of heterozygous effects on glucose accumulation were also evident. There are three main processes that may have influenced the greater muscle glucose accumulation observed in AA genotypes. These processes are discussed below:

1) *Glucose delivery to the skeletal muscle:*

Glucose delivery and uptake into the muscle is influenced by an exercise-induced increase in blood flow and the arteriovenous glucose difference (Ritcher & Hargreaves 2013). No study to the author's knowledge has examined the influence of FTO rs9939609 allelic variants on exercise-induced changes to either of the aforementioned factors. Blood flow was not measured in the current study; thus, we cannot exclude whether a difference in the exercise-induced increase in blood flow existed between allelic variants of FTO. As expected, an elevated plasma glucose concentration was evident in the early stages following high intensity exercise due to an imbalance between glucose supply and utilisation; a more rapid decline in utilisation than supply (Calles 1983). In response to exercise, a predominately linear

relationship exists between plasma glucose concentration and glucose uptake in muscle, indicating that changes in plasma glucose concentration can translate almost proportionally to changes in muscle glucose uptake (reviewed in detail by Rose & Ritcher 2005). However, plasma glucose concentrations were similar between FTO genotypes in response to high intensity exercise. Additionally, neither genotype nor time influenced plasma glucose concentrations in the early stages of recovery following low intensity exercise, and thus it is also unlikely that alterations in plasma glucose concentration would be responsible for the greater muscle glucose accumulation observed for AA genotypes following this protocol.

2) *Glucose transport into the sarcoplasm:*

Glucose uptake into the sarcoplasm depends on the skeletal muscle expression of GLUT4 (an insulin and contraction regulated glucose transport isoform) (Charron et al. 1989), which is increased following exercise and can facilitate post exercise glucose uptake (McCoy et al. 1996). It may be speculated that individuals homozygous for the FTO risk A-allele have a greater GLUT4 transporter expression and translocation in response to exercise compared to non-risk T-allele carriers, which could facilitate greater glucose uptake. Indeed, a recent investigation showed that *Fto* knockdown suppresses GLUT4 expression *in vitro*, whilst *Fto* overexpression elevated the expression of GLUT4 (Jiao et al. 2016). However, Jiao et al. (2016) detected this association in pre-adipocytes, and thus it is unknown if a similar effect would occur in skeletal muscle. Furthermore, no investigation has explored whether risk variants of FTO have higher levels of FTO protein, or higher levels of GLUT4 expression. As elevated PGC1α protein levels have been found to enhance GLUT4 gene expression and insulin sensitivity in cultured muscle cells (Michael et al. 2001), exercise stimulated improvements to glucose transport and insulin sensitivity may be

acutely regulated, among other factors, by PGC1α. Patient with T2DM have elevated levels of FTO mRNA, and interestingly have reduced levels of PGC1α (Bravard et al. 2011). Whether this inverse relationship has any impact on glucose transport or insulin sensitivity requires further investigation. Additionally, Bravard et al. (2011) did not investigate whether a relationship existed between elevated FTO in the skeletal muscle of T2DM patients and GLUT4 expression in this tissue. Other studies that have explored relationships between FTO and metabolic genes (including GLUT4 and PGC1α) have done so in response to nutritional stressors (Grunnet et al. 2009*b*; Jia et al. 2012), where changes observed in the skeletal muscle expression of these genes likely occur as a secondary response to insulin stimulation and not necessarily due to a direct relationship with FTO. An increased translocation of GLUT4 to the cell membrane would expectedly facilitate a greater uptake of glucose from plasma into skeletal muscle, and as discussed prior, plasma glucose concentrations were similar between genotypes in the current investigation. However, this does not rule out that an FTO genotype specific influence on GLUT4 expression or activity may have existed in response to exercise. The measurement of whether skeletal muscle GLUT4 and/or PGC1α levels and activity, or elements of the insulin-signalling cascade, are associated with the observed genotypic difference in muscle glucose accumulation was not investigated in the current study and remains warranted.

3) *Glucose metabolism within the cytosol:*

During exercise recovery, the resynthesize of muscle glycogen is a high metabolic priority (Kimber et al. 2003) and no study to date has examined whether the rate of this resynthesis varies between allelic variants of the FTO rs9939609 polymorphism. As such, it can be speculated that in the process of phosphorylating glucose for

glycogen conversion there may be an interim period where glucose accumulates during exercise recovery in homozygous risk A-allele carriers (in comparison to TT genotypes) whilst the demand to produce ATP via glycolysis is slowing down. Furthermore, in a previous observation of increased intramuscular glucose concentrations during maximal dynamic exercise (97% VO_{2max} for 15 mins), it was proposed that glucose accumulated in the muscle secondary to hexokinase inhibition (Katz et al. 1986); causing the rate of glucose phosphorylation and utilisation to be lower than the rate of glucose uptake. Katz et al. (1986) proposed that the inhibition of hexokinase might have occurred due to elevations in glucose-6-phosphate in response to increased muscle glycogenolysis (Katz et al. 1986). To date, no study has examined whether differences in hexokinase activity exists across genotype alleles of the FTO rs9939609 polymorphism. If an inhibition of hexokinase did happen to be the cause of greater muscle glucose accumulation in AA genotypes, then an associated elevation in muscle glucose-6-phosphate might also have been expected in the early stages following exercise (as this is proposed as the limiting metabolite for hexokinase action (Berg et al. 2002)). However, glucose-6-phosphate was not identified as a VIP metabolite in the current data set and as such was not selected for further analysis.

As respiratory gas exchange was only examined throughout exercise and not during the passive recovery period in the current study, it is unknown whether a genotypic difference in muscle glucose metabolism in the early stages following exercise would have been reflected in RER and substrate utilisation measurements. The data collected for these variables throughout exercise did not revealed differences across genotype alleles in the ability to utilise glucose or fat as an energy source during either exercise

intensity. No other investigation to date has examined substrate utilisation between FTO rs9939609 genotypes in response to exercise, and a previous study has not detected an association between FTO genotypes and substrate oxidation in either preprandial or postprandial conditions (Grunnet et al. 2009*b*). The current study also showed the ability of the skeletal muscle to cope with changes to metabolic demand (adjust fuel oxidation based on alterations in supply) during both exercise conditions to be similar between FTO genotypes.

In the current study, elevated levels of muscle fumarate/succinate were observed at 10 minutes following high intensity exercise, and lower levels at 90 minutes following low intensity exercise (compared to pre exercise measurements). Furthermore, a trend for genotype specific elevations in these metabolites was also observed in response to high intensity exercise, with AA and AT genotypes found to have a 56.6% and 53.7% increase in muscle fumarate/succinate in the early stages of exercise recovery (compared to pre exercise measurements), respectively. TT genotypes also displayed an increase in muscle fumarate/succinate; albeit at a much smaller amount (22.0%). Previous literature has shown elevated levels of fumarate and succinate to inhibit FTO protein function (Gerken et al. 2007), however it is unknown whether high intensity exercise-induced disruptions to energy flux in the TCA cycle may alter FTO function via elevations in these metabolites, particularly in risk A-allele carriers. FTO protein was measure in chapter 6 and the relationship between fumarate/succinate will be discussed in more detail then.

5.6 Concluding Remarks

The current investigation indicates that metabolic perturbation with exercise can distinguish genotypic differences in skeletal muscle metabolism that are not detectable in a basal state. Furthermore, a relatively homogeneous group enabled the examination of metabolic differences without the confounding effects of extraneous variables associated with disease and obesity. Whilst multivariate analysis of metabolomic data was unable to detect a clear metabolic profile for the differing allelic variants of the FTO rs9939609 polymorphism, the model used is likely to be limited by an inability to differentiate data at individual time points and by using a relatively small sample size. Despite this, a novel finding in this investigation was that individuals homozygous for the risk A-allele had significantly higher muscle glucose than individuals homozygous for the non-risk T-allele at 10 minutes post exercise, regardless of intensity. Further research should investigate why greater glucose accumulation occurs in the skeletal muscle of AA genotypes in the early stages following exercise, and how this may influence FTO at a cellular level. At this stage, it is unknown whether the exercise-stimulated accumulation of muscle glucose observed in AA genotypes is a factor that could contribute to, or attenuate, disease susceptibility in the long term. The ability of high intensity exercise to induce a trend for elevated muscle fumarate/succinate in risk A-allele genotypes, compared to the TT genotype, also warrants further investigation to determine whether this metabolic alteration is associated with genotypic differences in FTO protein function.

CHAPTER SIX

The influence of exercise intensity on skeletal muscle FTO gene and protein expression, and protein function, between variants of the FTO rs9939609 polymorphism

6.1 Abstract

Recent meta-analysis has shown that the association between FTO risk allele and the odds of obesity can be attenuated by approximately a third in physically active adults, suggesting a possible relationship between exercise and FTO protein function. The purpose of this study was to examine changes in skeletal muscle FTO expression and protein function following high and low intensity exercise and determine whether these changes are genotypic specific. Twenty-eight apparently healthy untrained males and females were genotyped for the FTO rs9939609 (T>A) polymorphism, prior to performing continuous isocaloric (400 kcal) cycle ergometer exercise on two separate occasions at 80% (HI) and 40% (LO) VO_{2peak}. Skeletal muscle biopsies were sampled from the vastus lateralis pre exercise, and at 10 and 90 mins post exercise. No significant interaction was detected for either exercise or genotype on FTO protein expression ($p > 0.05$). A main effect for time was observed for FTO mRNA expression following HI intensity exercise ($p = 0.003$), and for m^6A methylation levels on RNA following both exercise protocols (HI, $p = 0.032$; LO, $p = 0.032$). A weak trend for a genotype by time interaction was found for FTO mRNA expression during the HI intensity exercise protocol ($p = 0.095$). Demethylation of m^6A on RNA was greater in individuals with TT genotypes during the early stages of recovery from

LO intensity exercise (p = 0.027), with no change in AA and AT genotypes (p > 0.05). No genotype by time interaction was detected for m^6A on RNA during the HI intensity exercise trial (p > 0.05). A positive correlation was observed between skeletal muscle FTO mRNA expression and glucose accumulation in AA genotypes (p = 0.049) during the HI intensity exercise trial. In conclusion, this is the first study to show that exercise can influence skeletal muscle FTO gene expression. Moreover, genotypic changes in RNA demethylation were also evident during the early stages of recovery from exercise. Finally, the association between muscle glucose and FTO mRNA levels may suggest an impact of FTO on carbohydrate metabolism.

6.2 Introduction

The regulation of gene expression is a fundamental process impacting on the ultimate phenotypic characteristics of tissues (Barrès et al. 2012). The methylation status of m^6A is a dynamic, reversible process that is one of the most common and abundant post-transcriptional modifications capable of influencing gene expression (Niu et al. 2013). Asymmetrical distribution of m^6A along mRNAs can influence the translation of gene transcripts via a reduction of methylation sites in the 5′ untranslated region (5′UTR) (Dominissini et al. 2012; Zhou et al. 2015). Furthermore, demethylation of m^6A on mRNA may also influence gene translation through various structural, splicing and transportation related effects (Dominissini et al. 2012; Meyer et al. 2012). In addition to catalysing the Fe^{2+}- and 2OG- dependent nucleic acid demethylation of 3-meT and 3-meU in single stranded DNA and RNA, respectively (Gerken et al. 2007; Han et al. 2010), FTO seems to be demethylator of m^6A on mRNA (Jia et al. 2011). The consequences of FTO-guided demethylation are unknown, but it is likely to affect the processing of pre-mRNA, other nuclear RNAs,

or both. In FTO mutation models, studies have shown that if FTO protein is reduced, m^6A on mRNA increases (Hess et al. 2013; Jia et al. 2011), and vice versa if FTO protein is increased (Jia et al. 2011; Zhang et al. 2015). The discovery that FTO functions as a cellular m^6A demethylase, and that levels of m^6A in target mRNAs are altered following changes in FTO activity in FTO mutant models (Meyer et al. 2012), provides evidence that FTO contributes to the onset of obesity and related diseases, however, the cellular pathways impacted are yet to be identified.

Pathways that control energy homeostasis and metabolism may be one way that altered levels of FTO contributes to cellular function. Elevated basal levels of FTO mRNA and protein have been reported in the skeletal muscle of T2DM patients, which is characterised by disruptions to muscle glucose uptake and utilisation (Bravard et al. 2011). The elevated levels of FTO and associated metabolic disturbances were independent of BMI, as obese individuals and healthy lean controls displayed lower levels of FTO. However, whether the metabolic disturbances are driving the elevated levels of FTO, or whether the FTO protein is driving the disturbances is unknown. In Chapter 5, high and low intensity exercise were used to elicit metabolic disturbances, which may change FTO levels. Exercise can disrupt the intramuscular milieu, influencing metabolites involved in glucose metabolism, including glycolytic and TCA cycle pathways. Alterations to TCA intermediates, in particular fumarate, have been found to modulate the FTO protein, with Gerken et al. (2007) demonstrating an inhibition of FTO function by elevated fumarate levels *in vitro*. Given exercise is known to significantly increase fumarate levels in the skeletal muscle (Gibala et al. 1997*a*; Gibala et al. 1997*b*), subsequent modulation of FTO function may also occur.

The aims of this study were twofold; Firstly, to measure whether difference in FTO mRNA and protein expression, and m^6A on RNA, exist in skeletal muscle. Secondly, to observe whether an acute bout of exercise influenced FTO expression and function (measured by m^6A methylation status) in the skeletal muscle. It was hypothesized that exercise would induce a greater elevation in skeletal muscle FTO mRNA and protein expression on variants encompassing the risk A-allele (AA and AT genotypes), and increased demethylation of m^6A on RNA, in comparison to individuals homozygous for the non-risk allele (TT genotypes). It was also hypothesized that the higher intensity exercise would induce greater perturbations to FTO expression and function than lower intensity exercise.

6.3 Methods

6.3.1 Participants

Skeletal muscle samples from the twenty-eight apparently healthy, untrained males and females who participated in the Victoria University Human Research Ethics Committee approved study (*HRETH 12/197*) (previously described in Chapter 5) were used for this investigation. Information regarding the participant consent process, exclusion criteria, genotyping procedures, body composition assessment (via DEXA), VO_{2peak} fitness testing, and experimental exercise trial protocols have been previously detailed in sections 5.3.1 – 5.3.5. The skeletal muscle samples collected in Chapter 5 (see section 5.3.7 for description) were snap-frozen in LN_2 at the time of collection and ultra-freeze stored at -80°C until used to analyse FTO mRNA and protein expression, and m^6A RNA methylation. These samples were collected pre exercise (0 mins), and at 10 and 90 mins throughout passive recovery (post exercise) during the HI (80% VO_{2peak}) and LO (40% VO_{2peak}) intensity exercise trials described in section 5.3.5.

6.3.2 Skeletal Muscle FTO mRNA Expression: RT-PCR

Total RNA was isolated from ~20 mg of frozen skeletal muscle, reverse transcribed, and cDNA synthesised as detailed in section 3.4.7.1. FTO mRNA expression was determined using a CFX96 Real-Time thermal cycler (Bio-Rad Laboratories, VIC, Australia). ß-Actin (ACTB) was used as an external control standard for each reaction due to its previous verification as a constitutively expressed "housekeeping gene" in human skeletal muscle following acute exercise (Willoughby et al. 2007) (see section 3.4.7.2 for full amplification details and Table 3.3 for mRNA sequences of the

oligonucleotide primers used). The relative expression of mRNA was assessed by determining the ratio between the C_T values of each target mRNA and the C_T of ß-Actin for each skeletal muscle sample obtained throughout the experimental exercise trials. The fold change in expression was then calculated using the fold change $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen 2001).

6.3.3 m^6A RNA Methylation Determination

The m^6A RNA methylation status of skeletal muscle samples was measured colorimetrically using an EpiQuick m^6A RNA methylation Quantification Kit as per manufacturer's instructions (see section 3.4.9 for full methodological details). Total RNA isolation in preparation for this analysis was performed as previously described in section 3.4.7.1. m^6A was quantified spectrophotometrically, and linear regression data (generated via a standard curve of positive control samples) was used to determine the m^6A concentration of each skeletal muscle sample.

6.3.4 Skeletal Muscle FTO Protein Expression: Western Blotting

Skeletal muscle samples were prepared for protein expression determination by using a cryostat to shave 25 x 30 µm sections of each sample prior to lysing these in homogenisation buffer (see section 3.4.8). The total protein concentration of each sample was determined colorimetrically using a RED 660 Protein Assay (G Bioscience, MO, USA), which is described in detail in section 3.4.8.1. Protein from muscle preparations were separated on 12% Tris-Glycine stain-free gels (Bio-Rad Laboratories, VIC, Australia) and probed with antibodies against FTO and Actin separately following transfer and membrane blockage, with both antibodies diluted in

5% skim milk powder in TBST (see section 3.4.8.2 for full methodological detail). The membrane was exposed chemiluminescently using Clarity Western ECL Substrate (Bio-Rad Laboratories, VIC, Australia) within a VersaDoc Imager (VersaDoc MP Imaging System, Bio-Rad Laboratories, VIC, Australia) (section 3.4.8.2). Densitometry was performed using Image Lab Software (Bio-Rad Laboratories, VIC, Australia) with FTO protein expression calculated relative to the expression of Actin.

6.3.5 Statistical Analysis

Data were analysed using SPSS software (IBM SPSS Statistics for Windows, Version 20, NY, USA). Results are expressed as mean ± SEM unless stated otherwise. The level of probability was set at $p < 0.05$. One-way ANOVA's were performed for subject characteristic data. Two-way ANOVA's with repeated measures (Sidak Bonferoni method) were used to calculate individual significance for each genotype with time as the within group factor and genotype as the between group factor. Where an interaction was detected, multiple comparisons with Tukey's post hoc tests were completed to identify the differences. Regression analysis was used to observe genotype specific relationships between skeletal muscle FTO mRNA expression and metabolites that had shown a significant interaction, or a trend towards significance, for a genotype by time interaction, as per the findings of Chapter 5. Linear regression and covariant analysis (ANCOVA) were used to determine the effect of age on allelic representation of dependent variables.

6.4 Results

6.4.1 Characteristics of the Study Participants

Similar participant characteristics were observed between FTO rs9939609 gene variants; AA (n = 10, 5F/5M) (weight, 74.5 ± 3.9 kg; height, 176.1 ± 2.8 cm; VO_{2peak}, 40.0 ± 1.4 ml.kg.min^{-1}), AT (n = 9, 4F/5M) (weight, 72.0 ± 2.6 kg; height, 169.9 ± 2.4 cm; VO_{2peak}, 39.0 ± 2.6 ml.kg.min^{-1}), and TT (n = 9, 6F/3M) (weight, 72.6 ± 4.2 kg; height, 172.1 ± 1.9 cm; VO_{2peak}, 37.9 ± 2.3 ml.kg.min^{-1}). Additional participant characteristic data, separated by genotype of the FTO rs9939609 polymorphism, are presented in Table 5.1 of the previous chapter. A genotype effect was detected for age (p = 0.038), with AT genotypes significantly older than TT genotypes (p = 0.019). The absence of an effect of age on FTO expression and m^{6}A RNA methylation levels between FTO genotypes was confirmed by ANCOVA (p > 0.05). There were no significant differences between FTO genotypes for fat mass, lean muscle mass, bone mineral content or blood pressure (p > 0.05). Workloads (W) performed during the HI and LO intensity exercise protocols were similar between genotypes (p > 0.05). Data on physiological markers and performance in relation to the HI and LO intensity exercise protocols have been previously reported in Chapter 5 (section 5.4.2), and as such this chapter will only report on skeletal muscle findings.

6.4.2 Effect of Exercise on FTO mRNA Expression in Skeletal Muscle

HI Intensity Exercise: A significant main effect for time was observed for FTO mRNA expression following HI intensity exercise at 80% VO_{2peak} ($p = 0.003$) (Figure 6.1 A). Subsequent pairwise comparisons revealed a significant increase in FTO mRNA expression from pre exercise to 10 mins post exercise ($p = 0.003$) and a very strong trend towards significance from pre exercise to 90 mins post exercise ($p = 0.053$). No genotype main effect was identified ($p = 0.487$). A weak trend for a genotype by time interaction was observed ($p = 0.095$).

LO Intensity Exercise: A weak trend for a main effect for time was observed for FTO mRNA expression following LO intensity exercise at 40% VO_{2peak} ($p = 0.097$) (Figure 6.1 B). No genotype main effect ($p = 0.925$), or genotype by time interaction ($p = 0.856$) was identified.

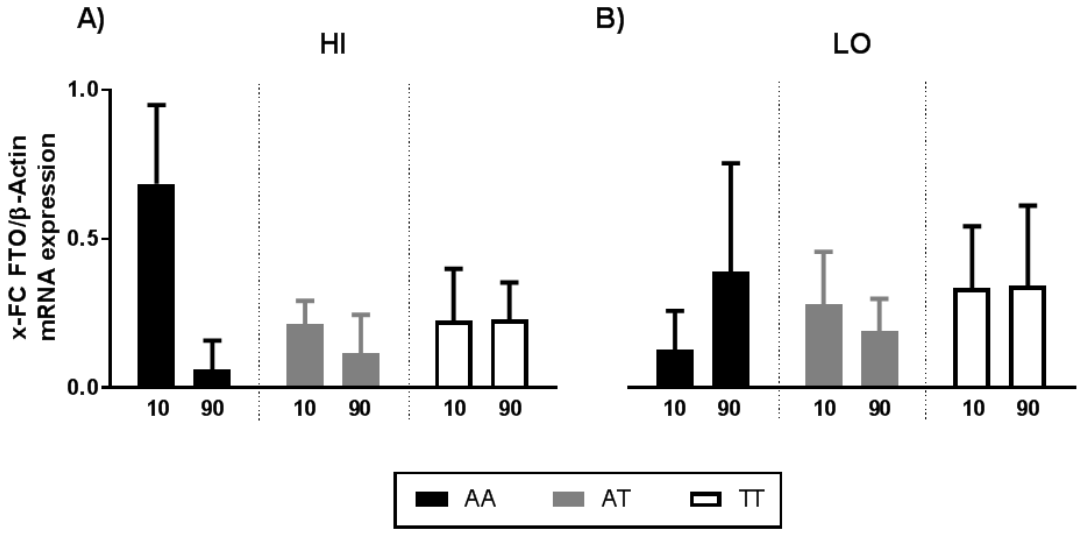


Figure 6.1 Relative expression of FTO mRNA normalised to β-Actin in human vastus lateralis skeletal muscle. Data is expressed as mean ± SEM of the fold change (x-FC) at 10 and 90 mins following A) 80% VO_{2peak} (HI) and B) 40% VO_{2peak} (LO) intensity exercise compared to pre exercise measurements. Data has been presented for all time points and in arbitrary units (AU) in Supplementary Figure S-6.1.

6.4.3 Effect of Exercise on Skeletal Muscle m^{6}A RNA Methylation

HI Intensity Exercise: A significant main effect for time was observed for m^{6}A on RNA following HI intensity exercise (p = 0.032) (Figure 6.2 A), with subsequent pairwise comparisons revealing a significant decrease in m^{6}A on RNA (demethylation) from pre exercise to 90 mins post exercise (p = 0.012). No genotype main effect (p = 0.537), or genotype by time interaction (p = 0.715) was identified.

LO Intensity Exercise: A significant main effect for time was observed for m^{6}A on RNA following LO intensity exercise (p = 0.032) (Figure 6.2 B). Subsequent pairwise comparisons revealed a very strong trend towards significance for decreased m^{6}A on RNA (demethylation) from pre exercise to 10 mins post exercise (p = 0.051), and a significant decrease in m^{6}A on RNA from pre exercise to 90 mins post exercise (p < 0.001) (Figure 6.2 B). A genotype by time interaction was identified for m^{6}A on RNA (p < 0.001), with individuals carrying the TT genotype demonstrating a significant decrease in m^{6}A on RNA at 10 mins post LO intensity exercise compared to pre exercise levels (p = 0.027). A main effect for genotype was observed (p = 0.006), with subsequent analysis revealing a strong trend for a greater reduction of m^{6}A on RNA (demethylation) in TT genotypes compared to AA genotypes (p = 0.058) at 10 mins post LO intensity exercise.

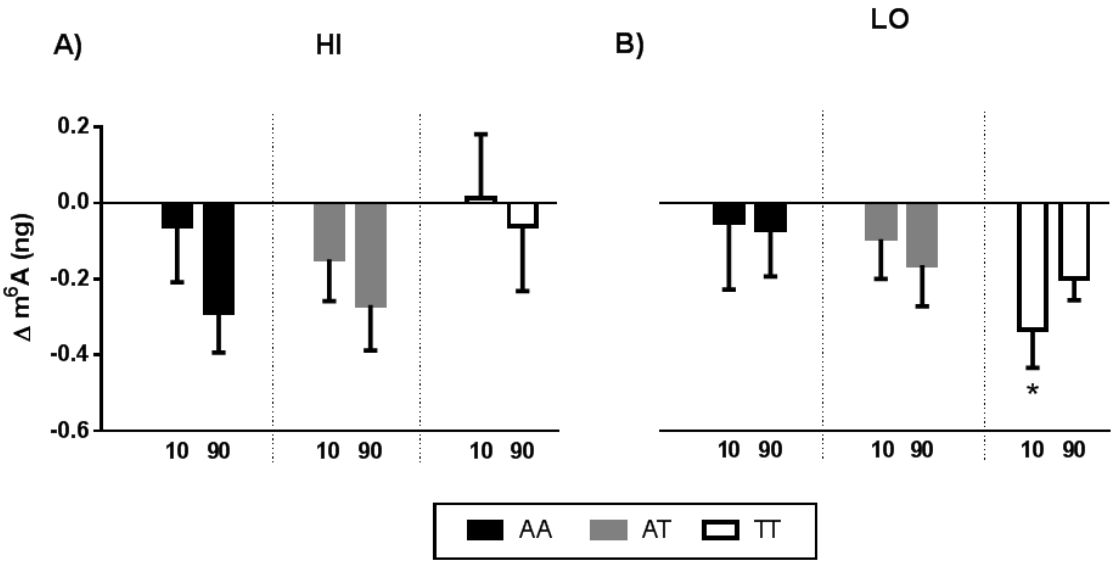


Figure 6.2 m^6A on RNA in the vastus lateralis muscle following isocalorically matched A) 80% VO_{2peak} (HI) and B) 40% VO_{2peak} (LO) intensity exercise trials. Data is expressed as mean ± SEM of the delta change from pre exercise measurements at 10 and 90 mins during passive recovery following A) HI and B) LO intensity exercise. * Significantly different from pre exercise measurements ($p < 0.05$).

6.4.4 Skeletal Muscle FTO Protein Expression in Response to Exercise

No main effect for time (p = 0.920), genotype main effect (p = 0.548), or genotype by time interaction (p = 0.217) was detected for skeletal muscle FTO protein expression in response to HI intensity exercise (Figure 6.2 B). Similarly, LO intensity exercise did not have a significant effect on skeletal muscle FTO protein expression, with no main effect for time (p = 0.510), genotype main effect (p = 0.632) or genotype by time interaction (p = 0.397) observed (Figure 6.2 C).

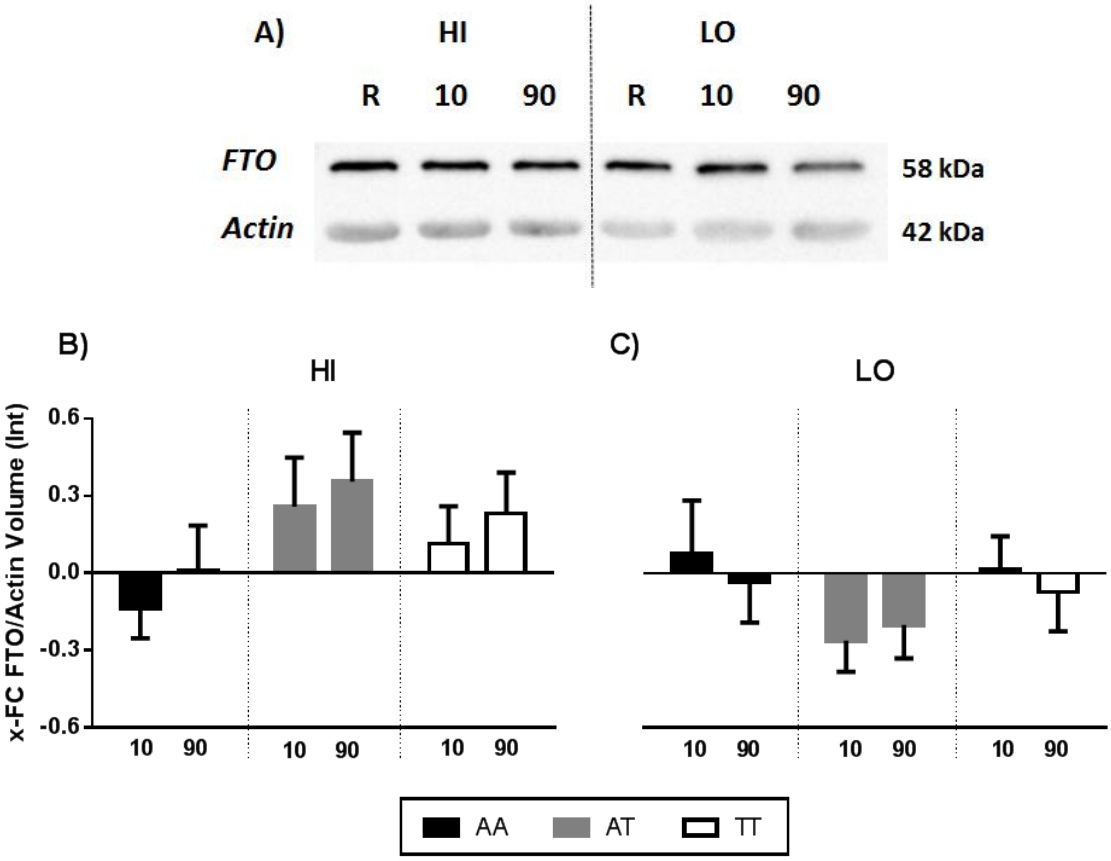


Figure 6.3 FTO protein expression in the vastus lateralis muscle following isocalorically matched A) 80% VO_{2peak} (HI) and B) 40% VO_{2peak} (LO) intensity exercise trials. A) Representative Western Blots of FTO and loading control Actin measured during pre exercise rest (R), and at 10 mins and 90 mins during passive recovery following isocaloric (400 kcal) stationary cycle exercise at 80% (HI) and 40% (LO) VO_{2peak}. B and C) Relative expression as indicated by densitometry (intensity, int) of FTO protein when normalised to Actin. Data is expressed as mean ± SEM of the x-FC at 10 and 90 mins compared to pre exercise measurements following HI and LO intensity exercise respectively.

6.4.5 Regression Analysis: Muscle Metabolites and FTO mRNA Expression

As a trend for a greater expression of FTO mRNA was observed for homozygous risk A-allele carriers following HI intensity exercise, regression analysis was conducted between FTO mRNA expression and muscle metabolites that had shown a significant interaction, or a trend towards significance, for a genotype by time interaction, as per the findings of Chapter 5.

A positive correlation was detected between skeletal muscle FTO mRNA expression and glucose levels (r^2 = 0.131, p = 0.049) in the skeletal muscle of AA genotypes during the HI intensity exercise trial, regardless of time (Figure 6.4). No relationship between skeletal muscle FTO mRNA expression and glucose levels was observed in the skeletal muscle of AT (r^2 = 0.007, p = 0.674) or TT (r^2 < 0.001, p = 0.985) genotypes during the HI intensity exercise trial.

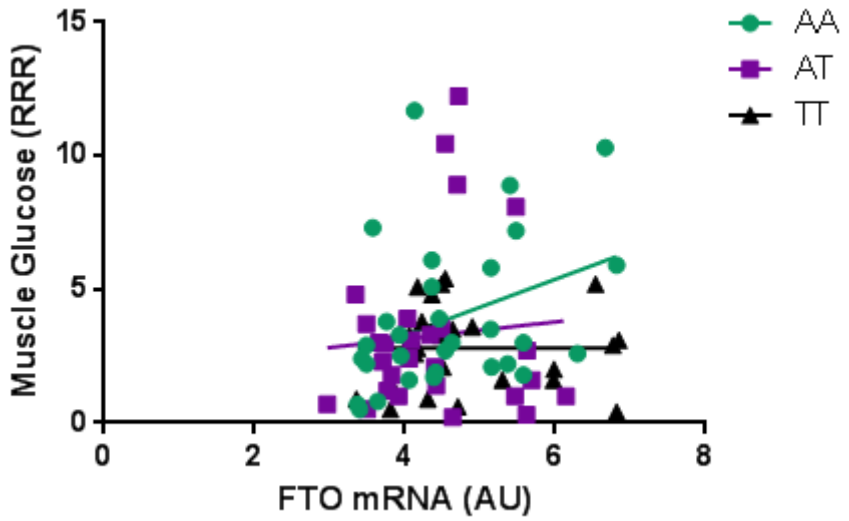


Figure 6.4 Correlation between glucose levels (presented as RRR to the ITSD ($^{13}C_6$-Sorbitol)) and FTO mRNA expression (normalised to ß-actin and presented as arbitrary units (AU)) in the skeletal muscle of participants during the HI intensity experimental exercise trial. AA, r^2 = 0.131, p = 0.049; AT, r^2 = 0.007, p = 0.674; TT, r^2 < 0.001, p = 0.985.

No statistically significant correlations were detected between FTO mRNA expression and fumarate/succinate levels in the skeletal muscle of the three FTO genotypes (AA, $r^2 = 0.084$, $p = 0.121$; AT, $r^2 = 0.009$, $p = 0.644$; TT, $r^2 = 0.001$, $p = 0.912$) during the HI intensity exercise trial (Figure 6.5).

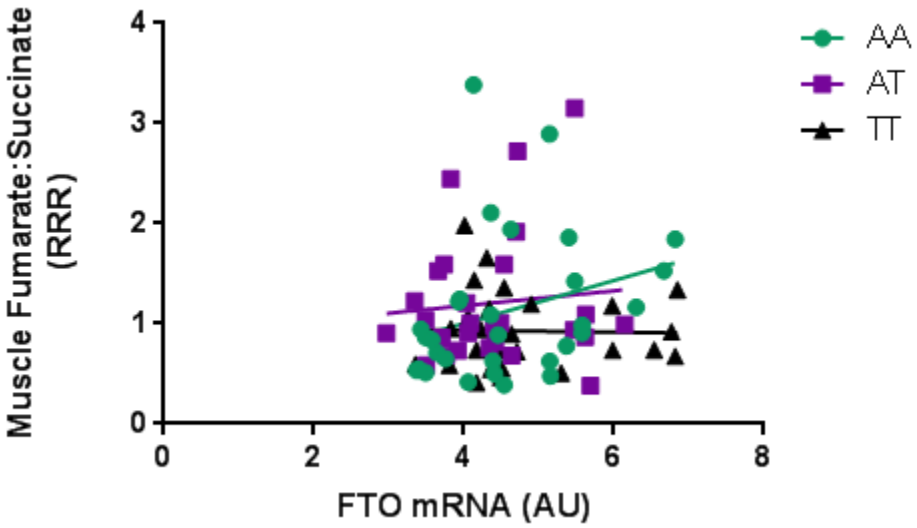


Figure 6.5 Correlation between fumarate to succinate ratio (presented as RRR to the ITSD ($^{13}C_6$-Sorbitol)) and FTO mRNA expression (normalised to ß-Actin and presented as arbitrary units (AU)) in the skeletal muscle of participants during the HI intensity experimental exercise trial. AA, $r^2 = 0.084$, $p = 0.121$; AT, $r^2 = 0.009$, $p = 0.644$; TT, $r^2 = 0.001$, $p = 0.912$.

6.5 Discussion

An acute up-regulation of skeletal muscle FTO mRNA was observed following high intensity exercise and individuals homozygous for the risk A-allele displayed higher FTO mRNA (albeit not significantly) in the early stages following high intensity exercise compared to those carrying the non-risk T-allele. FTO mRNA expression was positively correlated with intramuscular glucose levels for AA genotypes, with no relationship identified for AT or TT genotypes. Low intensity exercise demonstrated significantly greater demethylation of m^6A in carriers of the TT genotype, with no significant changes in AA and AT genotypes. Exercise intensity did not impact on FTO protein levels, which suggests that a singular acute metabolic stressor may not be sufficient to stimulate differences in FTO expression between allelic variants or a longer recovery time is required for protein formation. Overall, exercise was found to have some modulating effect on FTO expression, however this was limited to mRNA levels.

Data from the current study suggests that neither high or low intensity exercise, nor genotype, influenced FTO protein expression levels within the skeletal muscle tissue. No other investigation to date has examined FTO rs9939609 genotypic changes in FTO protein expression in response to exercise, or any alternate metabolic stimulus. In the present study, FTO rs9939609 risk and non-risk variants displayed similar basal levels of FTO protein. This novel finding highlights that those with risk variants of the FTO rs9939609 polymorphism may not have higher levels compared to those that have the non-risk alleles. Only one previous investigation has examined skeletal muscle FTO protein expressions, finding comparable levels in lean and obese individuals, and elevated levels in T2DM patients (Bravard et al. 2011). However,

Bravard et al. (2011) did not separate participants based on allelic variants of FTO, with the current investigation the first to do so. It is interesting that risk variants of FTO have a robust association with the odds of obesity, and that any link between T2DM and FTO occurs as an indirect result of FTO influencing BMI (Frayling et al. 2007), yet it is patients with T2DM who have elevated FTO protein levels and not obese individuals. This may indicate that a change in FTO function, rather than FTO expression levels, is the predominant contributor to phenotypic outcomes. Despite no significant changes in FTO levels following exercise, it cannot be ruled out that changes may be occurring following the 90 minutes of passive recovery, with previous research demonstrating changes in the expression levels of other proteins in the muscle to occur 3+ hours after isocaloric (400 kcal) exercise at 80% and 40% VO_{2peak} (i.e. calcium-dependent transcription factor, CREB) (Egan et al. 2010). Furthermore, repetitive metabolic stimuli, such as those obtained through exercise training, are generally required for physiological adaptations to occur, including alterations to protein content and activity (Egan et al. 2013). Thus, an acute metabolic stimulus of a single exercise bout, as opposed to chronic repetitive stimuli (such as that obtained from exercise training) may not impact the level of FTO protein expression. Furthermore, the partial eta-squared for FTO protein was found to be of a small size when performing high and low intensity exercise data analysis ($\eta^2 = 0.003$ and $\eta^2 = 0.027$ respectively), which suggests that a greater sample size may be required to detect potential exercise-induced changes to FTO protein expression levels.

Measurement of m^6A methylation on RNA was used as an indirect indicator of FTO protein function. Demethylation of m^6A on RNA occurred following both the high

and low exercise bout when not differentiating genotypes. When taking allelic variation into consideration, low intensity exercise was found to acutely influence m^6A demethylation at the RNA sites of TT genotypes, with a significant demethylation of m^6A on RNA observed at 10 minutes into recovery from pre exercise measurements and AA genotypes. No significant changes were observed following the high intensity exercise bout. However, there was a 3.7% and 9.6% and 17.2% and 17.3% increase in demethylation in AA and AT genotypes, respectively, at 10 minutes and 90 minutes post exercise, respectively, whereas the TT genotype showed a 1.1% and 4.7% increase in methylation at the same time points. It is not readily apparent as to why there were intensity dependent changes in demethylation of m^6A. The changes observed following the low intensity trial may indicate suppression of FTO function in individuals carrying the risk A-allele, whilst facilitating demethylation in the non-risk TT genotypes.

The observed changes in m^6A demethylation following high and low intensity exercise in the current chapter, in addition to the changes in muscle metabolites observed in Chapter 5, are contrary to what we would expect. High intensity exercise results in higher levels of fumarate and succinate than what is observed in a basal state (Gibala et al. 1997*a*; Gibala et al. 1997*b*), and high levels of these TCA intermediates can inhibit the function of FTO (Gerken et al. 2007). In contrast, Chapter 5 found the fumarate succinate ratio to not be influenced by low intensity exercise. Thus, if low intensity suppressed FTO function in variants encompassing the risk A-allele then this may suggest that other metabolites or alternate inhibitors of demethylation may have influenced methylation levels. Interestingly, in the acute stages following the high intensity exercise protocol an elevated fumarate to succinate

ratio was evident (predominately due to increased fumarate). This elevation was particularly evident in risk A-allele carriers, yet the high intensity exercise trial is where greater demethylation occurred for these genotypes; albeit not significant. It should be highlighted that the current study used m^6A methylation status of RNA as a non-specific measurement of FTO protein function, and that this method is unable to delineate whether demethylation occurred from FTO alone or due to other factors known to contribute to methylation status in response to exercise. Thus, it is possible that demethylation in response to high intensity exercise may have been affected by additional factors that can influence methylation status, including the expression of miRNAs (which can influence the binding of methyltransferases), or the activity of other demethylating genes such as ALKBH5 (Chen et al. 2015; Zheng et al. 2013). As the m^6A residues and miRNA binding sites within the 3' UTR are close to each other (Meyer et al. 2012), future research is warranted into the examination of whether FTO demethylates directly in response to exercise or via a potential interaction with miRNA pathways.

Another novel finding of this investigation was that high intensity exercise induced an acute and significant increase the expression of FTO mRNA during the recovery period, with no genotypic specific changes evident. However, AA genotypes did show a 0.69-fold increase in FTO mRNA expression at 10 minutes post exercise, whilst comparatively, FTO mRNA expression only increased by 0.21-fold and 0.23-fold for AT and TT genotypes respectively. Generally, elevated mRNA during recovery from acute exercise correlates with the synthesis of respective proteins (Perry et al. 2010), which could suggest that FTO protein may increase over a longer term. To date, only one study to date has examined whether differences in skeletal

muscle FTO mRNA expression exist between allelic variants. Although this was following nutritional stimuli, no genotype specific interactions were reported (Grunnet et al. 2009*a*; Grunnet et al. 2009*b*). However, as metabolic disturbances differ between nutritional and exercise stimuli these results may not be comparable to the current study. Notwithstanding, exposure to a nutritional stimuli (EHIC) has previously been found to increase the skeletal muscle expression of FTO mRNA, in parallel with metabolic genes PGC1α and GLUT4 (Grunnet et al. 2009*b*), suggesting that FTO may also be a nutritionally regulated metabolic gene. The elevated expression of skeletal muscle FTO mRNA observed following high intensity exercise, independent of genotype suggests that FTO is stimulated by exercise. This supports the possibility that FTO may be a metabolic gene, as skeletal muscle levels of GLUT4 and PGC1α are also known to become up-regulated in the short-term following high intensity exercise (Hood et al. 2011; Gibala et al. 2009). Additionally, fibre type specific changes may explain why there were elevated muscle FTO mRNA levels in the high intensity exercise trial, and not the low intensity exercise trial. Fast twitch fibres are utilised over slow twitch fibres as the intensity of exercise increases, and known metabolic genes such as GLUT4 have been shown to have a greater up-regulation in fast twitch fibres than in slow twitch fibres following high intensity exercise (at up to 85% of predicated maximum heart rate) (Stuart et al. 2010).

As metabolic genes are involved in the control of metabolism and work to restore metabolic functions upon environmental changes correlation analyses were preformed to determine whether relationships existed between FTO mRNA expression and metabolites showing a genotype specific change in Chapter 5. A positive correlation was found between muscle glucose and skeletal muscle FTO mRNA of AA genotypes

during the high intensity exercise trial. No correlation was found for FTO mRNA and muscle glucose for AT or TT genotypes. Whilst the results of this study support a relationship between elevations of FTO mRNA and glucose accumulation in the skeletal muscle, contradictory data exists. An increased expression of skeletal muscle FTO mRNA has previously been observed in T2DM patients, whom as part of their condition have impaired muscle glucose utilisation, when compared to healthy controls (Bravard et al. 2011). Additionally, the expression of FTO mRNA was not acutely regulated by either insulin or glucose in skeletal muscle following clamp methods (Bravard et al. 2011). On the contrary, skeletal muscle FTO mRNA expression has shown positive associations with glucose oxidation rates in response to an EHIC nutritional stimulus; however, this effect was not influenced by allelic variants of FTO (Grunnet et al. 2009*b*). Furthermore, this study did not examine the expression or activity of PGC1α or GLUT4, and thus it is unknown whether these genes play a role in the FTO mRNA associated accumulation of muscle glucose. Further work is needed to understand the relationship between skeletal muscle FTO mRNA expression and the accumulation of muscle glucose in response to environmental stressors. In particularly, it is important to elucidate whether this is a mechanism by which FTO risk variants may contribute to the metabolic complications previously associated with elevated FTO (Bravard et al. 2011).

6.6 Concluding Remarks

The findings from the current study are the first to show that skeletal muscle FTO mRNA is acutely regulated by exercise, and may have an allele specific response. Furthermore, this was the first study to use exercise to explore whether genotypic differences existed in the expression and function of the FTO protein in the muscle. Neither exercise, nor genotype, was found to influence FTO protein expression levels, however this may be limited by the singular design of the exercise bout. Low intensity exercise was able to exert a genotype-epigenetic specific demethylation response, with greater demethylation evident in TT genotypes in the early stages following exercise compared to baseline levels and that of the AA genotype. It is speculated that low intensity exercise may suppress the demethylation function of FTO in risk A-allele carriers, whilst high intensity may have a counter wise effect. Interestingly, a positive correlation between skeletal muscle FTO mRNA expression and glucose accumulation of AA genotypes was observed during the high intensity exercise trial, with no relationship between these variables detected for AT or TT genotypes. Whilst the mechanisms influencing this association are yet to be determined, this finding may provide a pathway for future research that explores the peripheral roles of FTO.

CHAPTER SEVEN

The use of metabolomics to monitor simultaneous changes in metabolic variables following supramaximal low volume high intensity exercise

Note: The study presented in the current chapter has previously been published by Danaher et al. (2016) in the journal '*Metabolomics*' (vol. 12). Permission has been granted by the publisher (Spinger) and all co-authors of this work to present this study in the current thesis. Minor alterations have been made to the presented studies format and body of literature.

7.1 Abstract

High intensity exercise (HIE) stimulates greater physiological remodelling when compared to workload matched low-moderate intensity exercise. This study utilised an untargeted metabolomics approach to examine the metabolic perturbations that occur following two workload matched supramaximal low volume HIE trials. In a randomised order, 7 untrained males completed two exercise protocols separated by one week; 1) $HIE_{150\%}$: 30 x 20 s cycling at 150% VO_{2peak}, 40 s passive rest; 2) $HIE_{300\%}$: 30 x 10 s cycling at 300% VO_{2peak}, 50 s passive rest. Total exercise duration was 30 mins for both trials. Blood samples were taken at rest, during and immediately following exercise, and at 60 mins post exercise. GC-MS analysis of plasma identified 43 known metabolites of which 3 demonstrated significant fold changes ($HIE_{300\%}$ compared to the $HIE_{150\%}$ value) during exercise, 14 post exercise and 23 at the end of the recovery period. Significant changes in plasma metabolites relating to lipid

metabolism (fatty acids: dodecanoate (p = 0.042), hexadecanoate (p = 0.001), octadecanoate (p = 0.001)), total cholesterol (p = 0.001), and glycolysis (lactate (p = 0.018)) were observed following exercise and during the recovery period. The $HIE_{300\%}$ protocol elicited greater metabolic changes relating to lipid metabolism and glycolysis when compared to the $HIE_{150\%}$ protocol. These changes were more pronounced throughout the recovery period rather than during the exercise bout itself. Data from the current study demonstrate the use of metabolomics to monitor intensity-dependent changes in multiple metabolic pathways following exercise. The small sample size indicates a need for further studies in a larger sample cohort to validate these findings.

7.2 Introduction

High intensity exercise (HIE), defined as exercise performed at ≥ 80% of an individual's VO_{2max} (Romijn et al. 1993), has gained increasing popularity. One reason for its popularity, especially in a time poor society, is the numerous health and fitness benefits that can be obtained from performing short bouts of exercise, albeit at a higher intensity (Gibala et al. 2012). Previous work has shown that when total workload is matched while exercise intensity (HIE versus endurance) and duration are varied (2.5 hours v. 10.5 hours for HIE and endurance exercise, respectively), similar physiological and cellular adaptations can occur (Gibala et al. 2006). Furthermore, our group recently confirmed these findings by demonstrating comparable changes in systemic markers of lipolysis and fat oxidation during the recovery period following workload matched low volume HIE and continuous (CON) exercise (Gerber et al. 2014). However, despite the two protocols being isoenergetic, plasma hypoxanthine accumulation and urinary purine base excretion was higher following HIE, compared

to CON exercise (Gerber et al. 2014). As these degradation products of ATP are considered an indirect indication of ATP loss from the muscle, it is suggested that the energy required for subsequent restorative processes (via intramuscular purine *de novo* replacement) may in part contribute to the negative energy balance that leads to fat loss often observed following long term HIE training programs (Gerber et al. 2014).

In addition, although HIE favours carbohydrate utilisation relative to fat as a fuel source during exercise, evidence suggests that the muscle adaptations that occur during recovery following HIE may induce greater fat oxidation post exercise when compared to low to moderate intensity bouts of exercise (Trapp et al. 2008; Tremblay et al. 1990; van Loon et al. 2001; Yoshioka et al. 2001). Higher relative fat oxidation post exercise could also be a contributing factor in the fat loss observed following HIE training. These findings and others (Gibala et al. 2012; Malatesta et al. 2009) indicate that training programs of high intensity and low volume may be very effective at stimulating metabolic disturbances that induce enhanced weight loss in the form of reduced adipose tissue, but also promote health and fitness benefits normally seen over the long term.

To explore the aforementioned concepts further, given most research in this area only compares HIE to submaximal exercise, our laboratory examined altered exercise intensity and subsequent passive rest periods at a supramaximal level to establish whether skeletal muscle metabolism could be further manipulated at higher exercise intensities. Using three isoenergetic supramaximal low volume HIE protocols (150%, 200% and 300% of VO_{2peak}) and selecting specific metabolites that relate to glycolysis

and purine metabolism, we showed no differences in plasma inosine, xanthine and uric acid following the 3 workload matched protocols, but significant differences in plasma lactate area under the curve calculations and urinary hypoxanthine excretion (data not shown). These findings indicate that a stimulus threshold may exist for specific metabolites when supramaximal intensity exercise is performed and going above this threshold may not induce any greater physiological change or further stimulate already maximal adaptations rates (i.e. a "biological ceiling") as proposed by Booth & Watson (1985). However, since research to date has been highly selective with respect to the metabolites monitored, changes in other important metabolites and pathways are likely to be missed.

Metabolomics, defined as "the quantitative measurement of the dynamic multiparametric metabolic response of living systems to pathophysiological stimuli or genetic modification" involves characterising and quantifying a large number of metabolites and other small molecules that are present in a biological sample at one time (Nicholson et al. 1999). As such, metabolomics can provide a metabolite "signature" or "footprint" of the relative concentrations of multiple metabolites that can better predict phenotypic changes from genotypes (Barabási & Oltvai 2004). In the area of HIE research, metabolomic technologies may help monitor metabolite changes concurrent with exercise, which may elucidate any relationships or trends for comparison. Moreover, by utilising an untargeted metabolomics approach, both the characterisation of core metabolome changes commonly covered in the targeted approach can be achieved, with the addition of detecting previously unknown or poorly characterised metabolites (Boughton et al. 2011).

The primary purpose of this study was to compare two workload matched supramaximal low volume HIE models using an untargeted metabolomics approach. It was hypothesized that despite the workload matched nature of the protocols, the higher supramaximal exercise intensity would induce greater metabolic perturbations in glucose metabolism during exercise and fatty acid and lipid metabolism during the recovery period, compared to the lower supramaximal exercise intensity. This investigation was also conducted independently as a pilot study to optimise metabolomics specific methodologies in plasma prior to this technology being used to analyse the skeletal muscle samples obtained in Chapter 5.

7.3 Methods

7.3.1 Participants

Participants believed to meet the eligibility criteria for this study were asked to provide written consent based on documents previously approved by the Victoria University Human Research Ethics Committee (*HRETH 10/12*) as per section 3.1. Seven healthy, recreationally active males (22.9 ± 5.0 yr; 81.6 ± 4.9 kg; 178.7 ± 4.5 cm; 50.0 ± 6.1 ml.kg.min^{-1} VO_{2peak}) volunteered to take part in this study, with participants excluded from participating within the project if they met the exclusion criteria detailed in section 3.2. Participants were asked to refrain from consuming caffeine and alcohol, and from undertaking strenuous exercise 24-h prior to attending all experimental trials. Additionally, participants recorded their dietary intake for 24-h before the first experimental exercise trial and were asked to replicate meals the day prior to the subsequent trial.

7.3.2 Preliminary testing

VO_{2peak} was determined approximately one week prior to the first experimental trial using a standard graded exercise protocol with initial workloads set specifically for a male cohort (see section 3.4.4.1 for details of this test). Data obtained from the graded exercise test was used to determine the individualised wattage each participant would be required to cycle at during subsequent experimental exercise trials in order to be representative of 150% and 300% of their VO_{2peak}. Prior to commencing experimental exercise testing, participants underwent a familiarisation session to become accustomed to the exercise protocols to be performed.

7.3.3 Experimental Exercise Trial Protocols

Generic details regarding experimental exercise testing procedures are described in section 3.4.4.2. Participants were asked to complete two supramaximal low volume HIE protocols (Figure 7.1) in a randomised order and separated by at least one week: 1) $HIE_{150\%}$: 30 x 20 s cycling at 150% VO_{2peak}, 40 s passive rest (348 ± 27 W); 2) $HIE_{300\%}$: 30 x 10 s cycling at 300% VO_{2peak}, 50 s passive rest (697 ± 54 W). Exercise trials were 30 mins in duration and workload matched (i.e. exercise intensity and the duration of cycling and rest intervals were manipulated to ensure participants cycled at 150% and 300% of their VO_{2peak}). Participants were encouraged to maintain a pedal frequency between 80 – 100 rpm during cycling intervals. HIE was preceded by a rest period and followed by 60 min passive recovery in a supine position. Experimental trials were conducted in the morning, approximately 10 – 12 h after the last meal in order to produce a basal state. Borg Scale ratings of perceived exertion (RPE 6 - 20 scale) were recorded at 15 min intervals throughout the exercise bouts to determine

which low volume HIE protocol was perceived to be the most physically demanding by the participants (see Table 3.2 for RPE scale details).

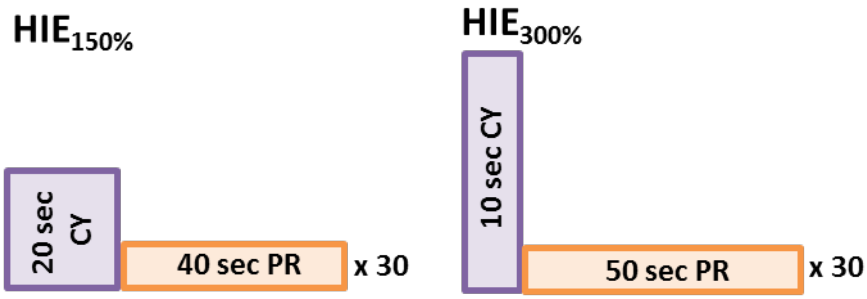


Figure 7.1 Workload matched protocol design. CY, cycle; PR, passive rest.

7.3.4 Blood Sampling and Preparation

Venous blood was collected from participants on each experimental exercise trial day as per sampling procedures detailed in section 3.4.2. Blood was sampled at REST (prior to exercise), 10 mins into exercise (EX10), immediately after exercise (EX30) and at the end of the 60 min recovery period (RC60). At each time point approximately 10 mls of venous blood was immediately placed into a lithium heparin tube (BD Vacutainer, BD Bioscience, NSW, Australia) and treated as per section 3.4.2. Plasma was decanted and stored at -80°C for later metabolomics and albumin analysis. Plasma albumin was measured using a commercially available BCG Albumin Assay Kit (see section 3.4.2.3) and used as an indirect marker and estimation of plasma volume changes during exercise (Maughan et al. 1985).

7.3.5 Metabolomics Analysis - GC-MS

7.3.5.1 Metabolite Extraction and Preparation

50 µL of each lithium heparin treated plasma sample was used for metabolite extraction in preparation for GC-MS (described in full in section 3.4.6.1). Each sample was spiked with 4% $^{13}C_6$-Sorbitol as an extraction ISTD. PBQC samples were created using 10 µl of supernatant from each extracted sample.

7.3.5.2 Instrumentation

The GC-MS system used (Figure 3.3), and instrument conditions set, for plasma metabolite analyses are described in section 3.4.6.3. The injection was operated in both a splitless (1 µl sample) and split (0.20 µl sample) mode to avoid overloaded chromatogram peaks.

7.3.5.3 Data Handling

Data handling procedures, including mass spectra verification, relative response ratio (RRR) analysis and peak verification procedures are detailed in section 3.4.6.4. Overloaded peaks (lactate, glucose, mannose, sucrose, fructose, urea and cholesterol) were analysed separately from the splitless mode. RRR data for each compound detected in each sample was normalised to the ISTD ($^{13}C_6$-Sorbitol). MetaboAnalyst 2.0 (Xia et al. 2012) was used to generate multivariate analysis pattern analysis models, with normalisation via a generalised log transformation applied to the data matrix to improve symmetry prior to multivariate analysis. Experimental data were additionally mapped on an author created metabolite network via VANTED V2.1.0.

The Human Metabolome Database (HMDB) (Wishart et al. 2013) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto 2000) database were used to assist in the development of this visual metabolite network.

7.3.5.4 Data Analysis and Statistical Methods

Multivariate Analysis: An in-house Metabolomics Australia Macro software (University of Melbourne, Australia) was used as an initial statistical measurement to determine x-fold change (x-FC), with Graphpad Prism 6.02 used to present x-FC results (GraphPad Software, CA, USA). Partial Least Squares Discriminant Analysis (PLS-DA) was performed on the whole set of metabolites (variables) at each time point to select those with a real discriminating power (SIMCA (version 14; Umetrics, Sweden). Metabolites corresponding to variable importance for projection (VIP) value $\geq$ 1.0 were classified as VIP metabolites and selected for further analysis. Two measurements of model quality were reported for PLS-DA: R^2Y and Q^2 representing, respectively, the goodness of fit (i.e., data variation) and the goodness of prediction, as estimated by cross-validation. $Q^2 \geq 1.0$ can be considered as a good predictor and < 0.5 as insignificant (Grootveld 2012; Wold et al. 1984). Cross-validation was used in each PLS-DA model to determine the number of components for subsequent univariate analysis and to avoid overfitting of the data. Normality of the distribution for VIP metabolites was tested using the Shapiro-Wilk test. When necessary, raw data were log-transformed to obtain normality. Normally distributed data were analysed using repeated-measures analysis of variance. If this analysis revealed any significant time effects and time x trial interactions ($P > 0.05$), paired t-tests were used to compare changes over time within trials, and differences between trials. Data that were not normally distributed after log transformation were analysed using the

nonparametric Friedman's test. If the result of this test was significant (P > 0.05), Wilcoxon's signed-rank tests were used to compare changes within trials, and differences between trials.

The paired data structure in the studies crossover multivariate data was suitable for multilevel PLS-DA (ML-PLS-DA) analysis. The data were analysed in parallel with the PLS-DA described above using a Matlab (MathWorks, USA, version 2015) routine provided by the Biosystems Data Analysis Group; Universiteit van Amsterdam (Westerhuis et al. 2010). Paired time points were assessed for consecutive samples and for EX10, EX30 and RC60, relative to REST. Default parameters were used for the n-fold component variables (1CV, 5; 2CV, 4; maximum number of PLS factors, 6; 2CV and CMV repeats (5 and 1000, respectively)).

Univariate Analysis: Two-way ANOVA's with repeated measures (Sidak Bonferoni method) was used to calculated individual significance for each VIP metabolite with time variable as the within group factor and exercise protocol variable as the between group factor. Where an interaction was detected, multiple comparisons with Tukey's post hoc tests were completed to identify the differences. Data are expressed as mean ± standard error of the mean (SEM) unless otherwise stated. The level of probability was set at $p < 0.05$

7.4 Results

Treatment of the chromatogram via AMDIS resulted in the detection of 55 metabolites, 12 of which had unknown identities (see Supplementary Table S-7.1 for parameters of known metabolites). Additional unknown compounds were detected, however only those peaks which demonstrated statistically significant changes between or within exercise protocols at time points other than baseline were selected for further analysis (see Supplementary Table S-7.2 for analysis of unknown metabolites). Paired crossover multivariate data models, 2D PLS-DA with Pareto Data Scaling was used to determine the within participant variation (Figure 7.2). PLS-DA ellipses represent 95% confidence intervals. At rest, PLS-DA modelling method demonstrated a similar (non-significant) metabolic profile between groups prior to commencement of each trial (P = 0.009), with very good validation metrics, including $R^2Y = 0.98$ and $Q^2 = 0.93$. At 10 mins post exercise, notable separation between trials was observed (P = 0.001), with good validation metrics, including $R^2Y = 0.77$ and $Q^2 = 0.74$. At 30 and 60 mins post exercise, a significantly clear separation between the two trials was identified (EX30, P = 0.005; RC60, P = 0.03), with moderate to high validation metrics, including $R^2Y = 0.88$ and $Q^2 = 0.70$ and $R^2Y = 0.90$ and $Q^2 = 0.76$ for 30 mins and 60 mins post exercise respectively.

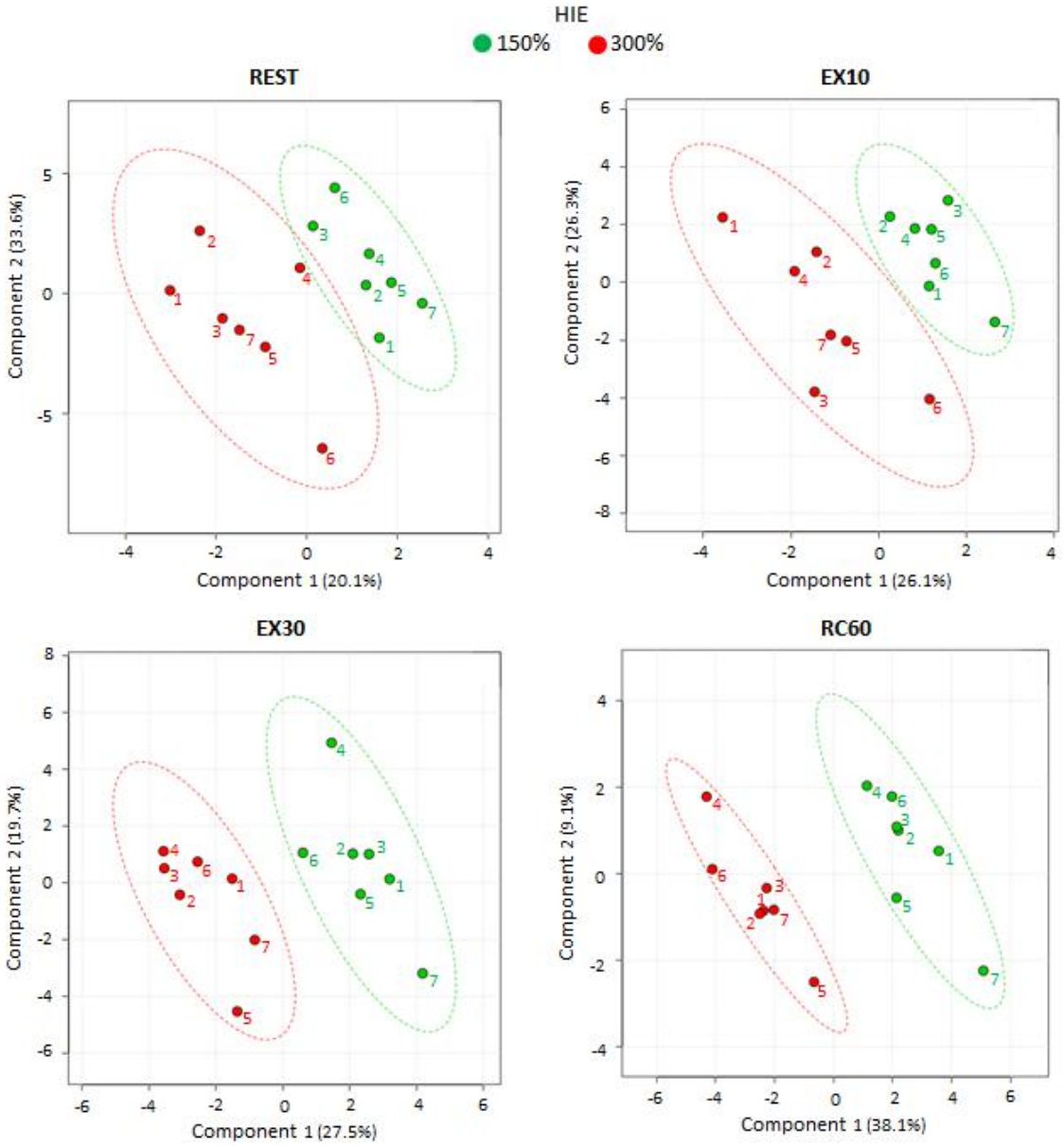


Figure 7.2 The 2D PLS-DA's of the within participant variation due to exercise intensity in stimulated data on the first two components from each time point. The red circles represent $HIE_{300\%}$ whilst the green circles represent $HIE_{150\%}$. Participants n = 1 - 7 are labelled. Ellipses are used to represent 95% confidence intervals. Component 1 describes the differences between the classes (exercise intensity) and Component 2 shows the within participant variation that is different between participants. Variances for each time point are indicated in brackets along each of the time points corresponding axis. The top 10 metabolite correlation coefficient scores in each time point are available in supplementary information (Figure S-7.1).

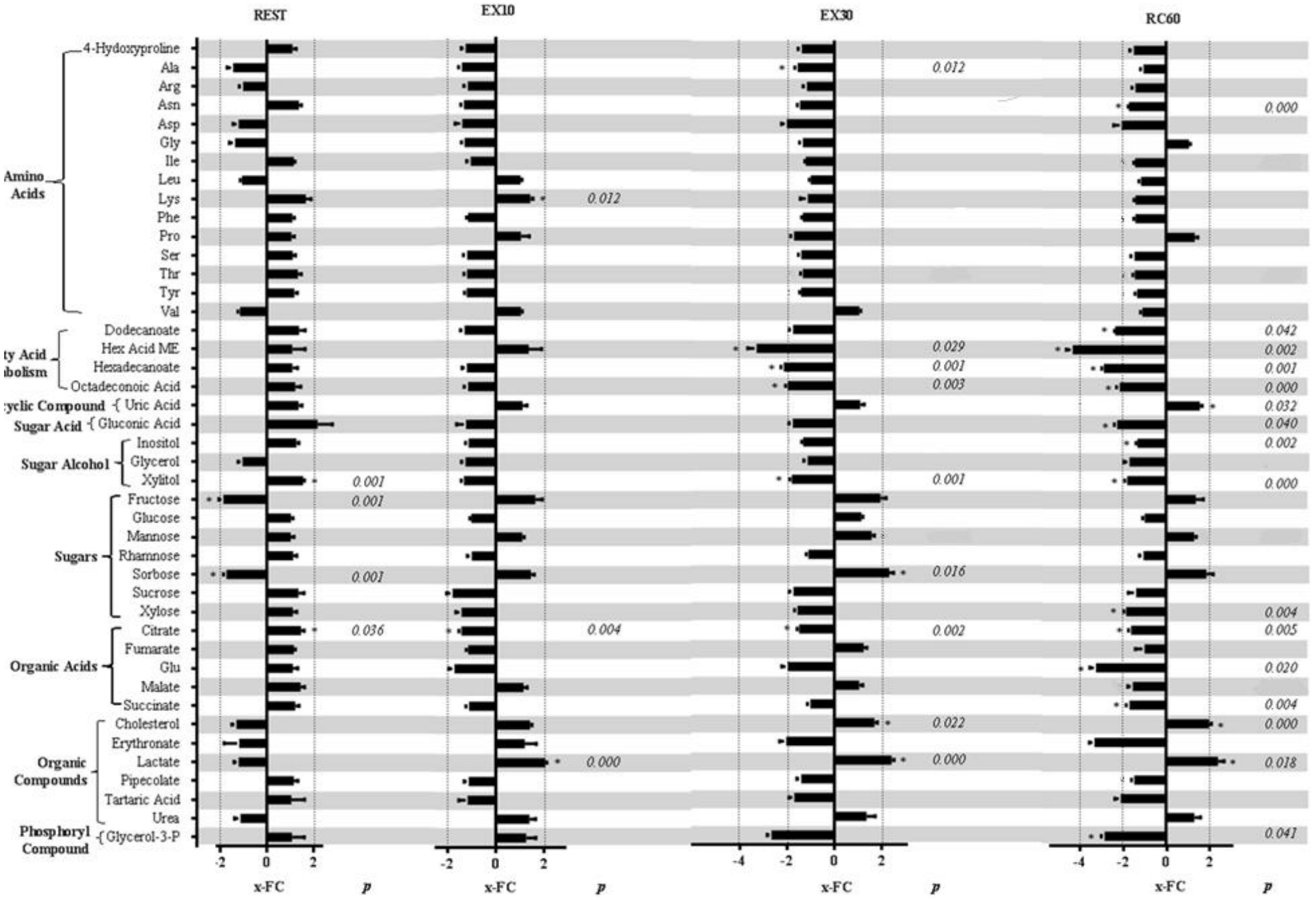
Correlation coefficient scores based on the weighted sum of the PLS regression identified the top metabolites with the overall greatest influence on the components at each time point (Supplementary Figure S-7.1). PLS-DA cross validation determined 25 metabolites in total with VIP scores value ≥ 1 for subsequent ANOVA analysis to determine which metabolites responded to exercise (Effect of Exercise Intensity (within group analysis)) and the different intensities (Effect of Exercise Intensity between groups) (Supplementary Table S-7.3 lists metabolites with VIP scores of ≥ 1 for each time point).

7.4.1 Effect of the Exercise Trial

Both exercise protocols ($HIE_{150\%}$ and $HIE_{300\%}$) elicited similar responses in heart rate with no differences observed during and following the exercise session ($p > 0.05$). Similarly, exercise-induced changes in plasma albumin concentrations were also comparable between trials at all observed time points ($p > 0.05$). A significant difference in RPE was shown at 15 mins and 30 mins following exercise commencement, with participants perceiving the $HIE_{300\%}$ condition "harder" when compared to the $HIE_{150\%}$ bout when considered on a numerical scale analysis and presented as median (interquartile range) (15 mins: $HIE_{150\%}$, 12 (10 – 13) (representing "Fairly light"), $HIE_{300\%}$, 15 (14 – 16) (representing "Hard") ($p = 0.003$); 30 mins: $HIE_{150\%}$, 13 (11 – 14) (representing "Somewhat Hard"), $HIE_{300\%}$, 16 (13 – 18) (representing "Hard") ($p = 0.020$)).

7.4.2 Effect of the Exercise Intensity (between group analysis)

Fold changes (x-FC) were calculated for each time point by comparing the RRR for each detected metabolite in the $HIE_{300\%}$ trial to the corresponding $HIE_{150\%}$ trial value (Figure 7.3). At rest, fructose and sorbose were significantly lower, whilst sugar alcohol xylitol and TCA intermediate citrate were significantly higher in the $HIE_{300\%}$ trial compared to the $HIE_{150\%}$ trial ($p < 0.04$). During exercise, lysine and lactate were significantly higher ($p < 0.02$), whereas citrate was significantly lower in the $HIE_{300\%}$ trial compared to the $HIE_{150\%}$ trial ($p < 0.02$). Immediately following exercise, sorbose, lactate and cholesterol were significantly higher ($p < 0.05$), whereas amino acid alanine, saturated fatty acids hexadeconoic acid methyl ester, hexadecanoate, and octadeconoic acid, sugar alcohol xylitol and TCA cycle intermediate citrate were significantly lower in the $HIE_{300\%}$ trial compared to the $HIE_{150\%}$ trial ($p < 0.05$). During recovery, amino acid asparagine, saturated fatty acids dodecanoate, hexadeconoic acid methyl ester, hexadecanoate, and octadeconoic acid, saccharide gluconic acid, sugar alcohols xylitol, sugar xylose, TCA cycle intermediates citrate, glutamate, succinate, and organophosphate glyercol-3-P were significantly lower in the $HIE_{300\%}$ trial compared to the $HIE_{150\%}$ trial ($p < 0.05$). Conversely, uric acid, cholesterol and lactate were significantly higher in the $HIE_{300\%}$ trial compared to the $HIE_{150\%}$ at RC60 ($p < 0.05$).



VICTORIA UNIVERSITY
MELBOURNE AUSTRALIA

Figure 7.3 x-FC of $HIE_{300\%}$ compared to the $HIE_{150\%}$ protocol. Black asterisks (*) indicate $p < 0.05$, p-value indicated only where there is a significant different between groups.

7.4.3 Effect of the Exercise Intensity (within group analysis)

Univariate analysis was used to determine to impact of supramaximal exercise on individual metabolites (Figure 7.4).

Plasma lactate was increased significantly during and at the end of exercise in both trials ($p < 0.001$), but remained significantly elevated during the recovery period in the $HIE_{300\%}$ trial only ($p = 0.004$). Malate was significantly increased during and at the end of exercise in both trials ($p < 0.001$) and remained significantly elevated during the recovery period following the $HIE_{150\%}$ trial only ($p < 0.02$). Glutamate was significantly decreased during the recovery period following the $HIE_{300\%}$ trial only ($p < 0.05$). Citrate was significantly increased during exercise, immediately post exercise and 60 mins post exercise in the $HIE_{150\%}$ trial ($p < 0.005$), conversely, during the $HIE_{300\%}$ trial, citrate was significantly decreased during exercise ($p = 0.042$), with no significant changes occurring post exercise.

The glucogenic amino acid asparagine was significantly lower at the end of exercise and remained significantly lower at 60 mins post exercise following the $HIE_{300\%}$ trial when compared to rest ($p < 0.05$). Alanine was significantly higher at 60 mins post exercise compared to rest ($p = 0.033$) following the $HIE_{300\%}$ trial, whereas ketogenic amino acid lysine was significantly lower at 60 mins post exercise following the $HIE_{300\%}$ trial ($p < 0.05$). During the $HIE_{150\%}$ trial, alanine was significantly increased immediately post exercise ($p = 0.011$). Saturated fatty acids octadecanoic acid and hexadecanoate, organic acid erythronate, and sugars xylitol and xylose were significantly lower post exercise and during the recovery period when compared to rest in the $HIE_{300\%}$ trial ($p < 0.05$), whereas saturated fatty acid dodecanoate was

significantly lower at 60 mins post exercise ($p = 0.034$). During the $HIE_{150\%}$ trial, only xylose significantly changed, with an increase at 60 mins post exercise ($p = 0.013$). Sugars sorbose and fructose were significantly elevated at the end of exercise during the $HIE_{300\%}$ trial ($p < 0.05$). Conversely, sorbose was significantly lower at the end of the recovery period following the $HIE_{150\%}$ trial ($p = 0.016$). Glucose remained constant during exercise and post exercise in both exercise trials ($p > 0.05$). Total cholesterol significantly increased during exercise and remained significantly elevated post exercise following the $HIE_{300\%}$ trial ($p = 0.002$). Conversely, total cholesterol was significantly decreased during the recovery period following the $HIE_{150\%}$ trial ($p < 0.001$), with no significant changes observed during and at the end of exercise.

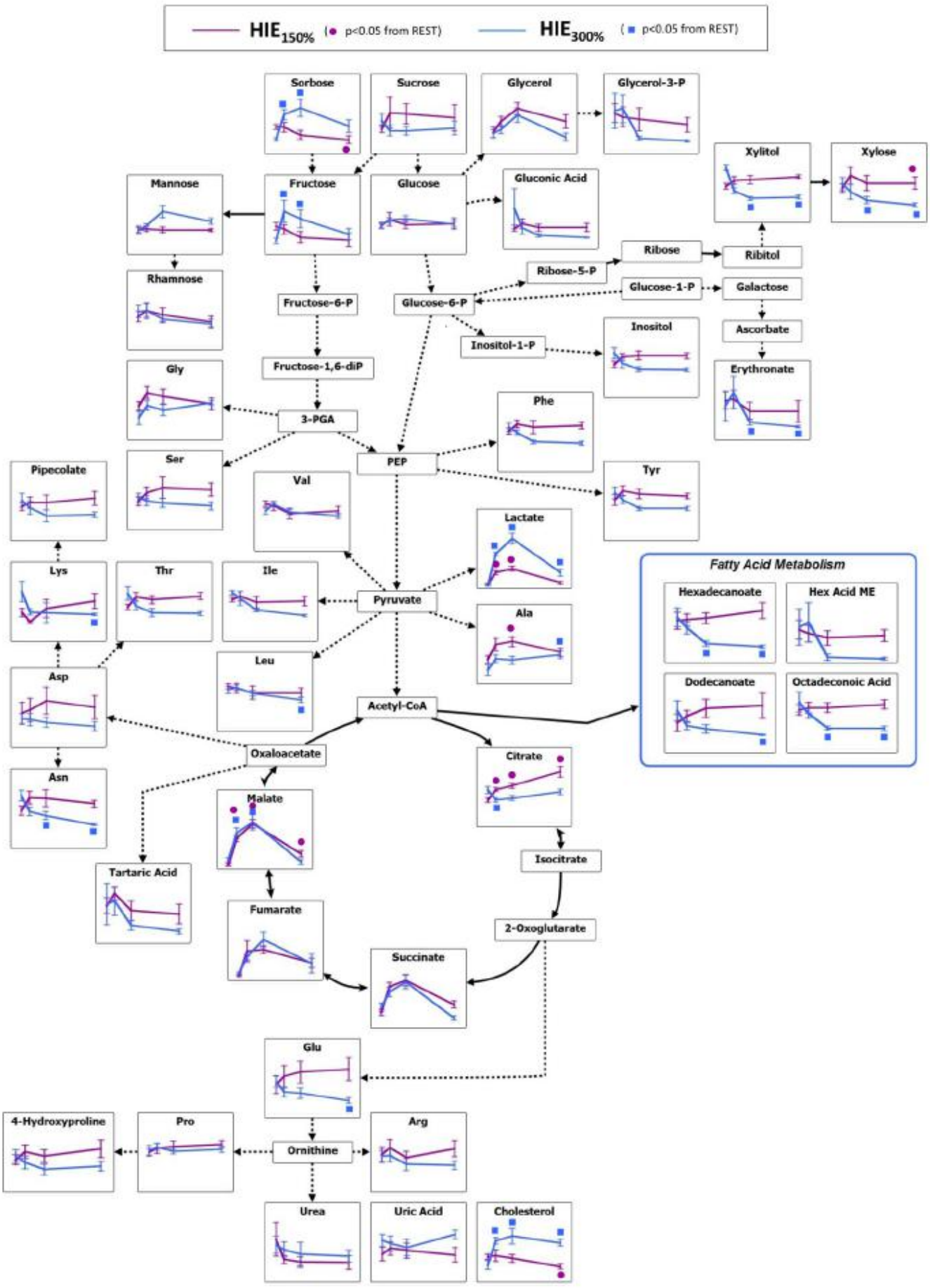

HIE150% (● p<0.05 from REST)
HIE300% (■ p<0.05 from REST)
Sorbose
Sucrose
Glycerol
Glycerol-3-P
Xylitol
Xylose
Mannose
Fructose
Glucose
Gluconic Acid
Ribose
Ribitol
Rhamnose
Ribose-5-P
Glucose-1-P
Galactose
Fructose-6-P
Glucose-6-P
Inositol
Ascorbate
Inositol-1-P
Gly
Fructose-1,6-diP
Erythronate
Phe
3-PGA
Ser
PEP
Tyr
Pipecolate
Val
Lactate
Lys
Thr
Ile
Pyruvate
Ala
Fatty Acid Metabolism
Hexadecanoate
Hex Acid ME
Leu
Dodecanoate
Octadeconoic Acid
Asp
Acetyl-CoA
Oxaloacetate
Citrate
Asn
Malate
Isocitrate
Tartaric Acid
Fumarate
2-Oxoglutarate
Succinate
Glu
4-Hydroxyproline
Pro
Arg
Ornithine
Urea
Uric Acid
Cholesterol

Figure 7.4 Metabolic pathway map for the two supramaximal low volume HIE protocols. $HIE_{150\%}$ is represented in purple, the $HIE_{300\%}$ is represented in blue, and is expressed as mean ± SEM for REST, EX10, EX30 and RC60 as bars move from left to right. Purple circles and blue squares represent significant changes within the $HIE_{150\%}$ and $HIE_{300\%}$ condition respectively ($p < 0.05$) when compared to REST, and are placed above or below the condition according to their increased or decreased relative response ratio (RRR). Metabolites are not to scale between each node.

The frequency histogram produced by the ML-PLS-DA analysis of paired time-points for the two exercise levels did not show a clear difference in the number of misclassifications (NMC), relative to PLS-DA for any of the comparisons examined (Figure 7.5). The red dot in each histogram shows the classification error (estimated number of misclassifications) for the model, relative to the permutations (blue). The prediction error (NMC = 0) is less than the permutations and similar for both ML-PLS-DA and PLS-DA, indicating that the statistical significance of the two methods is similar. The lower panel in the figure shows the rank product ($RP^{1/20}$) with the lowest RPs most strongly associated with the treatment effect. In rank order, the five metabolites contributing most at each interval were, respectively, 0-10: malate, succinate, alanine, lactate, glutamine; 10-30: malate, gluconic acid, arginine, fumarate, lactate; 30-60: malate, lactate, aspartate, rhamnose, glycerol; 0-30: malate, alanine, succinate, fumarate, lactate; 0-60: alanine, isoleucine, citrate, leucine, rhamnose. The lack of any clear difference between the ML-PLS-DA and standard PLS-DA analysis may be due to the small sample size in the current study and the relatively noisy data when comparing results from different time points.

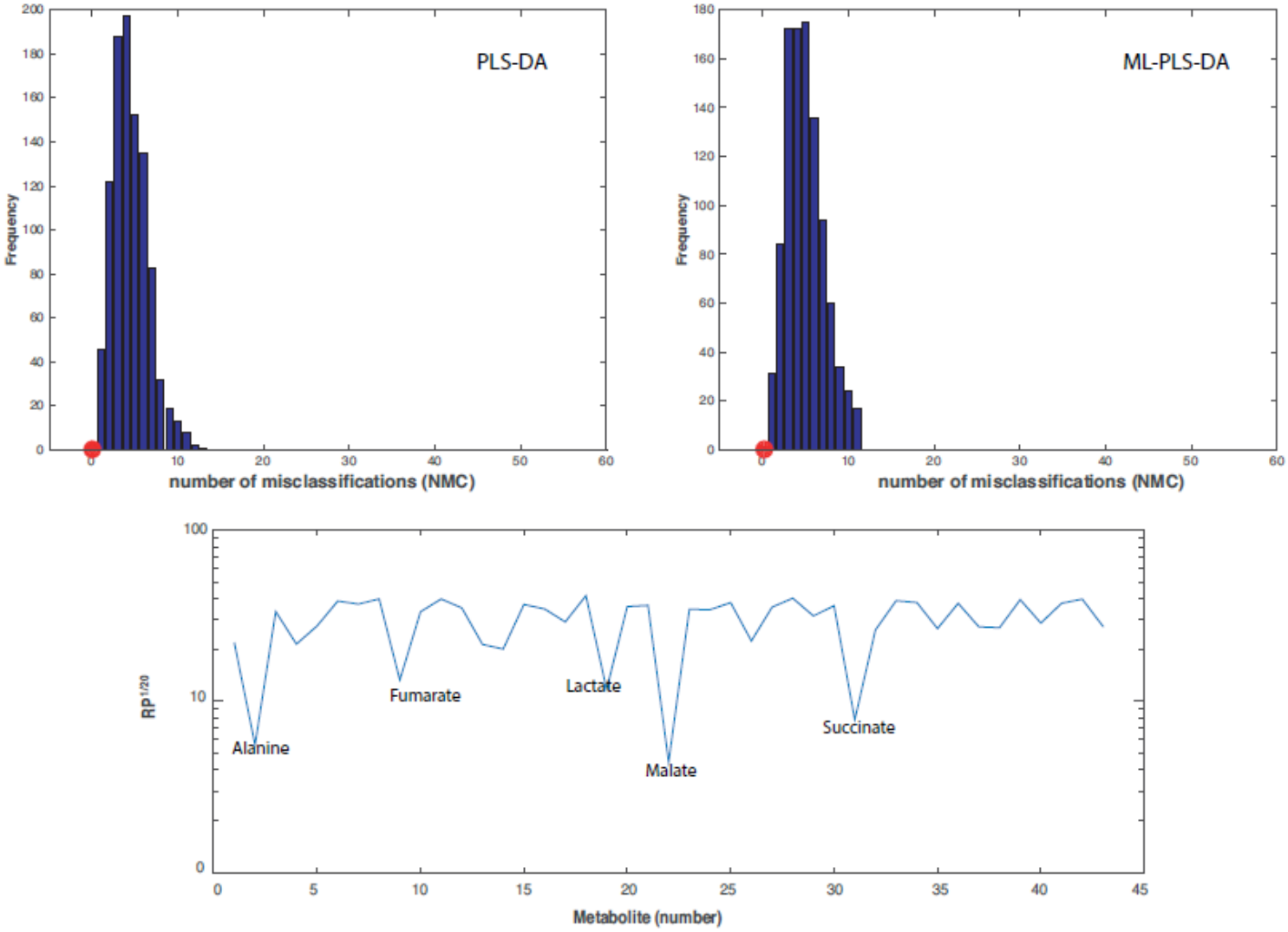


Figure 7.5 The red dot in each histogram shows the classification error (estimated number of misclassifications) for the model, relative to the permutations (blue).

7.5 Discussion

The current study used an untargeted metabolomics approach to monitor parallel changes of multiple metabolic perturbations induced by two workload matched supramaximal low volume HIE protocols, with different work to rest ratios. The variation in intensity of these protocols was designed to elicit substantial metabolic disturbances whilst minimising the time between efforts for energy replenishment in order to stimulate a physiological response. The major finding of the study is that despite the two protocols being matched for total work, the $HIE_{300\%}$ trial produced greater metabolic perturbations compared to the $HIE_{150\%}$ trial. Furthermore, changes were more pronounced during the recovery period rather than during exercise and were specific to metabolites of the glycolytic pathway, as well as fatty acid and lipid metabolism; suggesting higher workload draws on cellular reserves more than liver and circulating energy levels. Data from the current study highlight the benefit of using metabolomic technology to examine exercise intensity-dependent changes in metabolic pathways that may contribute to the proposed health and fitness benefits often observed with HIE training.

The lack of any clear difference between the ML-PLS-DA and standard PLS-DA analysis may be due to the small sample size in the current study and the relatively noisy data when comparing results from different time points. Malate was the predominant metabolite identified as significant in the ML-PLS-DA at all time intervals and lactate was significant between all time points. Additional metabolites participating in the TCA were also shown to significantly influence the model (succinate, citrate, and fumarate). Metabolites involved in other pathways that were identified in the PLS-DA and also identified by the ML-PLS-DA include amino acids

involved in the restoration of hepatic glucose metabolism (particularly alanine and glutamine) and branched amino acids typically degraded during exercise (isoleucine and leucine).

HIE leads to a reduction in skeletal muscle glycogen levels and in recovery, the metabolic priority of the muscle is to restore glycogen stores (Malatesta et al. 2009). Numerous studies have also demonstrated an increase in whole-body fat oxidation after glycogen depleting exercise, indicating both a shift towards greater fat oxidation and restoration of the plasma bicarbonate pool (Bielinski et al. 1985; Gerber et al. 2014; Kiens & Richter 1998). Despite these findings, little attention has been paid to the role of lipid utilisation during the recovery process (Bielinski et al. 1985; Kiens & Richter 1998). Following exercise, sources of fat are obtained from circulation (fatty acids, chylomicrons, very low density lipoproteins (VLDL)), or within the muscle fiber itself in the form of lipid droplets adjacent to the mitochondria (Kimber et al. 2003). Though intramuscular triglycerides (IMTG) may contribute to the lipid source for fat oxidation, it is likely that plasma fatty acids are the predominant contributor to increased fatty acid oxidation post exercise (Kimber et al. 2003). Indeed, Kimber et al. (2003) demonstrated a rapid decline in non-esterified fatty acids (NEFAs), early in the recovery period following approximately 85 minutes of varied intensity exercise. In the current study, plasma concentrations of fatty acids dodecanoate (C12 - also known as lauric acid), hexadecanote (C16 - also known as palmitic acid), hexadecanoic acid methyl ester (C17 - also known as palmitic acid, methyl ester), and octadecanoic (C18 - also known as stearic acid), significantly decreased during the 60 minute recovery period following the $HIE_{300\%}$ trial compared to the $HIE_{150\%}$ trial. Few studies have used a metabolomics approach to examine changes in NEFAs. Peake and

colleagues (2014) used a targeted metabolomics approach to investigate changes in metabolites following two isoenergetic bouts of interval HIE (80% VO_{2max}) and moderate intensity continuous exercise (65% VO_{2max}) in well trained cyclists and triathlete males. Contrary to the findings of the current study, interval HIE at 80% VO_{2max} increased specific NEFAs myristic acid (C14:0), decanoic acid (C10:0), dodecanoic acid (C12:0), heptadecenoic acid (C17:1) and palmitoleic acid (C16:1) after exercise, with some of these fatty acids remaining elevated for two hours post exercise (Peake et al. 2014). Additionally, moderate intensity continuous exercise at 65% VO_{2max} also increased these specific NEFAs. It is clear that intensity (60% and 80% VO_{2max} v. 150% and 300% VO_{2peak}) of the protocols and training status (trained versus recreationally active) of the participants used in the Peake et al. (2014) study, compared to the current study, are different and as such may explain the contrasting changes in specific plasma fatty acids following exercise and during the recovery period. Furthermore, participants in the Peake et al. (2014) study consumed breakfast prior to exercising which may have impacted on the metabolic response to exercise. Notwithstanding, the significant reductions in plasma free fatty acids observed in the current study could demonstrate their usage as a primary source of fuel during the recovery period as confirmed by other studies (Friedlander et al. 1998; Kimber et al. 2003). It is understood that changes in plasma free fatty acids during exercise are dynamic and recovery following exercise represents a balance between fatty acid release from adipose tissue and fatty acid uptake into skeletal muscle and liver (Mulla et al. 2000). Given no food was ingested post exercise in the current study; an insulin-mediated inhibition of lipolysis was unlikely to occur, although a catecholamine-induced lipolytic effect post exercise cannot be ruled out. It is not readily apparent however, as to why only the higher supramaximal HIE trial showed a decrease in

plasma free fatty acids. It could be that both trials increased fatty acid mobilisation during recovery and that the higher intensity exercise showed preferential utilisation of these saturated fatty acids. However, RER, an indicator of fatty acid oxidation was not measured post exercise and is thus a limitation in the current study.

Exercise training promotes beneficial effects on health by reducing total cholesterol, low-density lipoprotein cholesterol (LDL-C) and very low-density lipoprotein (VLDL) levels, and triglycerides (TG) (Bellou et al. 2013; Mulla et al. 2000; Tsekouras et al. 2008). Contrary to the majority of studies that have used moderate to high intensity protocols of continuous nature (60 - 90% VO_{2max}) to study the impact on total cholesterol and lipid metabolism, the current study utilised a supramaximal intermittent intensity protocol of 150% VO_{2peak} and 300% VO_{2peak}. The current results demonstrated contrasting outcomes, with the 300% VO_{2peak} protocol increasing total cholesterol during exercise and throughout the recovery period, whereas the 150% VO_{2peak} protocol decreased total cholesterol during the recovery period only. Although there is a lack of comparable studies, one study did examine the impact of a bout of supramaximal intensity exercise performed at 115% VO_{2max} (Lira et al. 2010). The results from this study showed no significant impact on serum total cholesterol or other markers of lipid metabolism (Lira et al. 2010). Given the conflicting exercise-induced changes in plasma lipid profiles (including total cholesterol), several studies have suggested that an "energy expenditure threshold" may exist and that higher levels of energy expenditure may be required to promote favourable changes in lipid metabolism (i.e. reduction in TG and the increase in HDL-C concentrations) following HIE (Aellen et al. 1993; Lira et al. 2009; Tsekouras et al. 2008). Indeed, the exercise session performed in the Lira et al. (2010) study generated 50% less energy

expenditure than their previous study which demonstrated a beneficial impact on lipid metabolism (Lira et al. 2009). Despite this, both protocols used in the current study were workload matched and thus similar energy expenditures would be assumed. On the other hand, exercise intensity was not the same between trials and this may have impacted hepatic regulation differently due to the higher energetic stress and higher levels of circulating catecholamines (Lira et al. 2012). A limitation to the present study was the measurement of only one form of lipid in the plasma and as a result changes in HDL-C and TG⁄HDL-C are unknown. Further research is needed to confirm the observed changes following the 150% and 300% VO_{2peak} exercise bouts, but also the impact of higher intensities on hepatic metabolism and serum lipid profiles.

The TCA cycle is the major final common pathway for oxidation of carbohydrates, lipids and some amino acids, resulting in the production of large amounts of ATP via oxidative phosphorylation. The TCA cycle flux is dependent upon the supply of acetyl units and activation of the three non-equilibrium reactions within the TCA cycle. Evidence suggests that an increase in the total concentration of the TCA cycle intermediates is imperative to increase and maintain TCA cycle flux during exercise (Peake et al. 2014). Measurement of plasma TCA cycle intermediates suggests that these metabolites can be released (or at least efflux) from the muscle as well as other metabolically active organs, such as the liver. The current analysis revealed significant increases in plasma TCA cycle intermediates (malate, succinate, and fumarate) in response to both supramaximal exercise intensities. Plasma citrate was also significantly increased in the $HIE_{150\%}$ trial, but was significantly decreased during the $HIE_{300\%}$ trial. These results are in accordance with a previous metabolomics-based

study investigating plasma changes with exercise in which accumulated levels of malate, fumarate and succinate were reported 60 minutes following exercise in healthy individuals (Lewis et al. 2010). This has also been confirmed in two recent studies using HIE protocols (Brugnara et al. 2012; Peake et al. 2014). There is some evidence to suggest that changes in TCA cycle intermediates are intensity dependent with previous investigations demonstrating greater changes during HIE compared to low-moderate exercise of similar duration and energy expenditure (Peake et al. 2014). The current study showed no difference between the two supramaximal bouts of exercise, which may indicate a potential intensity threshold in which no further changes can occur above a certain exercise intensity. Interestingly, despite no differences between groups evident during exercise (excluding citrate), there were differences during the recovery period with both malate and fumarate remaining significantly elevated following the $HIE_{150\%}$ trial only, compared to rest. These results and those observed for fatty acid and lipid metabolism, suggest intensity of exercise, especially at supramaximal levels, and may have a greater impact on specific metabolites during the recovery period than during the exercise bout itself.

The minimal effect of both exercise trials on the appearance of plasma amino acids during exercise in the present study may suggest that the liberated amino acids at the beginning of exercise were used for synthesis of TCA cycle intermediates and glutamine synthesis and thus, were not released from the muscle. During the recovery period restoration of hepatic glucose metabolism is a priority and amino acids (particularly alanine), pyruvate and lactate assist in that process (Brugnara et al. 2012; Mourtzakis et al. 2006). Further, glutamine and alanine may stimulate a significant rise in arterial blood glucose concentration during recovery from exercise (Brugnara

et al. 2012; Mourtzakis et al. 2006; Peake et al. 2014). Indeed both lactate and alanine were increased post exercise following both trials and may have contributed to the maintained glucose levels observed.

7.6 Concluding Remarks

The utilisation of an untargeted metabolomics approach provides researchers with the ability to examine a wider range of metabolic interactions and potential pathways. However, this is not without its limitations and is restricted by the current mass spectra library database. Thus, the ability to identify all detected metabolites and full interpretation of all metabolic changes in the present study was determined by the limitations of this database at the time. With continual updating of the library database with new metabolites, the scope of this technology will be expanded and will be more informative in the future. Additional limitations in the current study may have been the relative small cohort studied and differences in some metabolites at rest, although most likely not due to a training/stress effect given the cross-over design of the study. As such, the need for further studies using larger cohorts are required to confirm and extend the current findings. In summary, this study demonstrated greater metabolic perturbations in fatty acid, lipid metabolism and glycolysis following a $HIE_{300\%}$ protocol compared to a $HIE_{150\%}$ protocol. These changes were more pronounced during the recovery period than during the exercise bout and highlight that future work should investigate the impact of supramaximal low volume intermittent HIE on different plasma fatty acids during exercise and recovery period. This study provides further support for the beneficial impact of supramaximal exercise on total cholesterol and other lipids, which may have positive health implications. Finally, given the contrasting effects observed during recovery from exercise between protocols, there is potential to explore different recovery techniques (i.e. passive versus active) with a metabolomics approach and examine how such differences impact on metabolite recovery.

CHAPTER EIGHT

Conclusions and Future Directions

Obesity and its related comorbidities represent a global public health burden, and account for a substantial portion of health care spending worldwide. Elucidating the mechanisms behind potential genetic influences (and subsequent metabolic responses) on obesity may assist in developing effective lifestyle and pharmacological interventions to avoid weight gain or alleviate the detrimental health consequences of this common condition. Since FTO was initially associated with obesity risk 9 years ago many clues as to its functions have been obtained through *in vitro* and *in vivo* investigations. These have suggested an apparent pleiotropic nature of FTO and highlight the complexity of linking genetic variants to specific cellular and metabolic mechanisms. Whilst the role of FTO in the hypothalamus has received much attention, knowledge on FTO's mechanisms in peripheral tissues have not been as well researched. This dissertation contributes to a further understanding of the influence of FTO in skeletal muscle and provides evidence to suggest that FTO may influence carbohydrate metabolism. However, whether this has a more chronic effect and contributes to FTO's association with obesity and T2DM requires further investigation. The main findings of this dissertation are listed.

1. Similar metabolic flexibility exists between allelic homologues of the FTO rs9939609 polymorphism, whilst overweight individuals have a slower metabolic flexibility compared to lean individuals.

Investigations into the biochemical characteristics of metabolic pathways may help identify mechanistic processes in circumstances where the function of a gene is unclear (Gieger et al. 2008). This concept was adopted in Chapter 4, where an acute nutritional stimulus was used to manipulate the metabolic environment and observe whether differences in metabolic flexibility and substrate oxidation existed between FTO variants. Whilst metabolic inflexibility was found to exist in overweight individuals compared to lean, no apparent differences between genotypes existed, independent of BMI and gender. This could suggest that FTO risk variants may not impair metabolic flexibility in response to glucose ingestion, or that the positive energy balance generated by the acute glucose load may not impact FTO activity. This has not been explored in previous studies. It is possible that the apparent absence of differences in metabolic responses between FTO allelic variants may be due to the singular nature of the OGL challenge. In other words, an acute manipulation of carbohydrate oxidation and storage pathways may be insufficient to instigate changes in FTO status or influence the adaptive role of FTO. Rather, longer-term repetitive dietary exposure may be necessary to influence changes in FTO levels and activity. Elevated basal levels of FTO in the skeletal muscle of patients with the lifestyle disease T2DM may support this concept, with changes in expression levels potentially reflecting long-term exposure to adverse dietary choices (e.g. excessive sugar intake) and/or metabolic disturbances (i.e. an impaired ability to utilise glucose and thus a depletion of nutrients).

2. No differences in metabolic parameters were observed between FTO genotypes following an acute positive energy balance (i.e. dietary exposure) in Chapter 4.

Thus, the utilisation of an exercise stress to create an acute negative energy balance environment and increased muscle metabolism was employed in Chapter 5. This investigation used isocalorically matched high and low intensity exercise, in addition to an untargeted metabolomics approach, to observe whether differences in skeletal muscle metabolic profiles existed between FTO genotypes. Prior to commencing this investigation, a study presented in Chapter 7 was undertaken to independently verify that an untargeted metabolomics approach could be used to examine a wide range of metabolic interactions and potential pathways not observed with targeted approaches. Additionally, the study presented in Chapter 7 confirmed that an untargeted metabolomics approach could be used to monitor intensity-dependent changes in multiple metabolites following exercise, detect poorly characterised metabolites and metabolites unable to be monitored using targeted approached (i.e. ELISA kits), and provide clues for altered metabolic pathway activity. As such, the untargeted metabolomics approach was deemed as an appropriate tool to be adopted in Chapter 5 to help identify potential metabolites or metabolic pathways influenced by FTO.

3. No clear metabolic profiles were detected for differing allelic variants of the FTO rs9939609 polymorphism in response to high and low intensity exercise. However, a difference in muscle glucose accumulation was observed between allelic homologues at 10 minute post exercise, regardless of intensity.

Acute high intensity exercise alters metabolism to a greater extent than exercise of low-moderate intensity, with the results presented in Chapter 5 providing further

support for this already well-established verdict. Perturbations elicited by high intensity exercise predominately influenced metabolites involved in carbohydrate and protein metabolism, including amino acids, sugars (mono- and disaccharides) and TCA cycle intermediates. Despite this, no distinct metabolic profiles were identified between allelic variants of the FTO rs9939609 polymorphism in response to high intensity exercise, nor the low intensity protocol. However, AA genotypes did display a higher accumulation of glucose in the skeletal muscle in the early stages following both high and low intensity exercise, when compared to their TT genotype counterparts. It is understandable that high levels of glucose would be observed in the early stages during recovery given that the metabolic priority of the muscle is glycogen resynthesis (which involves increased glucose uptake). In the high intensity trial, AA genotypes may have displayed higher intramuscular glucose levels if they exercised harder indicating greater glycogen depletion, which would lead to enhanced glucose accumulation compared to the alternate allelic variants. This could be argued, as AA genotypes expended 400 kcal faster than the AT and TT genotypes, and thus may have exercised harder; albeit not significant. However, this notion may not explain why AA genotypes also showed high intramuscular glucose levels following low intensity exercise (with similar levels to the high intensity trial), as the lower intensity protocol would be expected to have a lesser impact on glycogenolysis. It was also proposed that a greater flux of glucose into the muscle following both high and low intensity exercise may have occurred as a consequence of elevated GLUT4 activation or levels in risk A-allele genotypes. No investigation to date has explored whether differences in skeletal muscle GLUT4 levels and activity, or that of PGC1α and elements of the insulin-signalling cascade (e.g Akt phosphorylation), exist for allelic variants of FTO in response to exercise. As these factors influence glucose

uptake they remain an avenue for future investigation. It was also speculated that hexokinase activity or the rate of glycogen resynthesis during exercise recovery may vary between allelic variants of FTO, as both factors contribute to glucose metabolism within the cytosol, and thus may also influence muscle glucose accumulation. Additionally, higher intramuscular glucose levels in AA genotypes could implicate a functional role of FTO. As such, the influence of exercise on FTO expression levels and function were examined in Chapter 6.

4. Neither acute exercise, nor genotype, influenced FTO protein expression.

However, it is unknown whether a change in FTO protein expression may have occurred in a timeframe longer than the 90 minutes post exercise observed in Chapter 6, or whether subsequent repetitive stimuli over time, such as that obtained through exercise training programs, would be required to see changes in FTO protein levels. Furthermore, whilst no obvious changes were observed for FTO protein levels, this measure does not rule out changes in FTO function. Demethylation of m^6A on RNA occurred following high and low intensity exercise. Low intensity exercise influenced a genotype specific demethylation response, with a greater demethylation evident in TT genotypes in the early stages following low intensity exercise compared to baseline levels and AA genotype. Based on these findings it was speculated that low intensity exercise may be able to suppress the demethylation function of FTO in AA genotypes. Alternatively, following high intensity exercise AA and AT genotypes had a greater demethylation of m^6A on RNA compared to TT genotypes, albeit not significant. It is interesting that following high intensity exercise, and not low intensity exercise, was where elevations of metabolites known to disrupt FTO function were observed in A-risk allele genotypes (particularly fumarate), and may

suggest that alternate metabolites or factors may have influenced FTO activity and methylation levels. This could include the high intramuscular glucose levels observed in AA genotypes with high intensity exercise, miRNA expression (which can influence the binding of methyltransferases), or the activity of other demethylating genes.

5. Exercise was found to have some modulating effect on FTO mRNA levels. High intensity exercise up-regulated skeletal muscle FTO mRNA expression after 90 minutes of recovery, whilst a trend for an up-regulation was observed following low intensity exercise.

Whilst mixed fibre type samples were examined in this study, this result may indicate that fast twitch fibres activated by higher intensities may have differences in FTO transcription compared with slow twitch fibres, and potentially greater levels. This concept is yet to be investigated, it is supported by findings that reveal fast twitch fibres become smaller with age (while the size of slow twitch fibres is much less affected) (Lexell 1995) and a decline in FTO mRNA to be age dependent (Grunnet et al. 2009*b*). Adding to this, if miRNAs were involved in determining methylation status in addition to FTO (as mentioned above) then this influence may also be confounded by fibre type. For example, expression levels of miR-206 vary depending on muscle type (McCarthy & Esser 2007) and have been implicated in muscle development, growth/adaptation, regeneration and muscle-related diseases (see review by Ma et al. 2015).

6. When separating mRNA results based on allelic variants of FTO, AA genotypes displayed a weak trend for higher FTO mRNA in the early stages following high intensity exercise compared to TT genotypes. Additionally, FTO mRNA expression was positively correlated with muscle glucose accumulation in the skeletal muscle of AA genotypes, with no relationship between these variables identified for AT or TT genotypes.

Previous findings of elevated FTO levels in T2DM patients (Bravard et al. 2011) suggest a role of FTO in defects of glucose metabolism. In the case of T2DM patients, FTO may become elevated over time in response to glucose deprivation within the internal environment in addition to characteristics of the disease state such as altered insulin signalling. In this dissertation, the accumulation of glucose in the muscle of individuals with the FTO risk variant may indicate that a metabolic aberration is preventing glucose from being metabolised, resulting in an internal state of glucose deprivation that FTO mRNA may be responding to. The singular and short-term nature of the exercise stimulus adopted in this dissertation could explain why FTO mRNA levels were only up-regulated acutely following high intensity exercise, whereas it is likely that elevated basal levels of muscle FTO observed in T2DM patients are a consequence of longer-term metabolic duress. Whilst further research is required to confirm this theory, it could point to FTO acting as metabolic gene or sensitive to glucose deprivation.

High intensity exercise results in acute increases in FTO mRNA levels in AA genotypes. This implies a potentially disadvantageous consequence of exercise as overexpression of *Fto* alters insulin signalling and enhances lipogenesis *in vivo* (Bravard et al. 2011) and causes a dose-dependent increase in adiposity in mice

(Church et al. 2010). However, the aforementioned studies were reported in basal conditions and there may be a differential effect by the internal milieu with exercise. The internal environment may be differentially managed when energy utilisation and flux is elevated. Research has shown that long-term maintenance for weight is best managed through a balance of high-energy expenditure versus a high-energy input (Mayer et al. 1956). This could help explain why the association between the risk A-allele and the odds of obesity is attenuated by ~30% in physically active adults (compared to those who are physically inactive) (Kilpeläinen et al. 2011), and why individuals with the AA genotype lose more weight than TT genotypes in response to diet/lifestyle interventions (Xiang et al. 2016).

An exercise training study, which allows for a longer observation period and repetitive stimuli, may unmask how longer-term manipulations to the internal environment may influence FTO. To date, no investigation has adopted an exercise training intervention to explore the peripheral and metabolic mechanisms of FTO. Determining how obesity risk variants influence an individual's response to longer-term exercise training can help tailor the prescription of effective and personalised exercise programs aimed at improving the metabolic outcomes. Further exploration in this avenue of research may provide genetically susceptible individuals with an insight into risk reducing behaviours that can be effective in prevention of obesity, and consequentially may offer a sense of control by demonstrating that genetic susceptibility is modifiable by lifestyle. Furthermore, the ability to reduce morbidity and mortality in a population who have increased odds of obesity, particularly through the prescription of individually tailored exercise training programs, could ultimately assist in alleviating the social, health and economic burden of this common disease.

Over the past decade the concept of personalised medicine has gained increasing popularity and involves genotyping individuals for several disease susceptibility polymorphisms identified by GWA studies, and tailoring nutrition, exercise or medical advice based on genotype profiles for better prevention or treatment outcomes (see review by Senekal 2012 for a thorough description of personalised medicine). Whilst this approach to health care has begun to emerge, it does not typically incorporate fundamental behavioural and environmental influences on obesity or disease risk outcomes. Thus, it remains ambiguous as to whether personalised medicine based recommendations via genotype screening would measurably improve disease prevention or treatment outcomes. To further the potential application of personalised interventions and medicinal approaches for better health, an in-depth understanding of genes associated with obesity and alternate diseases (including their molecular function, expression, pathways and interactions) is necessary. Whilst this dissertation contributes to the understanding of the influence of FTO in skeletal muscle, it also opens the door for future research, such as how FTO may interfere with nutrient metabolism (e.g. carbohydrate metabolism through insulin PKB signalling), relationships between FTO variants and expression levels with metabolic and mitochondrial genes, and the comparative effect of repetitive exercise stimulus as opposed to acute stimuli on FTO outcomes. By continuing to improve our knowledge on FTO and its metabolic mechanisms it may be possible to implement novel and personalised therapeutic targets in the future.

REFERENCE LIST

Adamska, A, Karczewska-Kupczewska, M, Nikołajuk, A, Otziomek, E, Górska, M, Kowalska, I & Strączkowski, M 2014, 'Relationships of serum soluble E-selectin concentration with insulin sensitivity and metabolic flexibility in lean and obese women', *Endocrine*, vol. 45, no. 3, pp. 422-429.

Aellen, R, Hollmann, W, & Boutellier, U 1993, 'Effects of aerobic and anaerobic training on plasma lipoproteins', *International Journal of Sports Medicine*, vol. 14, no. 7, pp. 396-400.

Albouaini, K, Egred, M, Alahmar, A & Wright, DJ 2007, 'Cardiopulmonary exercise testing and its application', *Heart*, vol. 93, no. 10, pp. 1285-1292.

Almén, MS, Jacobsson, JA, Moschonis, G, Benedict, C, Chrousos, GP, Fredriksson, R & Schiöth, HB 2012, 'Genome wide analysis reveals association of a FTO gene variant with epigenetic changes', *Genomics*, vol. 99, no. 3, pp. 132-137.

Andreasen, CH, Stender-Petersen, KL, Mogensen, MS, Torekov, SS, Wegner, L, Andersen, G, Nielsen, AL, Albrechtsen, A, Borch-Johnsen, K, Rasmussen, SS, Clausen, JO, Sandbæk, A, Lauritzen, T, Hansen, L, Jørgensen, T, Pedersen, O & Hansen, T 2008, 'Low physical activity accentuates the effect of the FTO rs9939609 polymorphism on body fat accumulation', *Diabetes*, vol. 57, no. 1, pp. 95-101.

Arts, HH, Doherty, D, van Beersum, SE, Parisi, MA, Letteboer, SJ, Gorden, NT, Peters, TA, Märker, T, Voesenek, K, Kartono, A, Ozyurek, H, Farin, FM, Kroes, HY, Wolfrum, U, Brunner, HG, Cremers, FP, Glass, IA, Knoers, NV & Roepman, R 2007, Mutations in the gene encoding the basal body protein RPGRIP1L, a nephrocystin-4 interactor, cause Joubert syndrome', *Nature Genetics*, vol. 9, no. 7, 882-888.

Aucouturier, J, Duché, P & Timmons, BW 2001, 'Metabolic flexibility and obesity in children and youth', *Obesity Reviews*, vol. 12, no. 5, e44-53.

Australian Bureau of Statistics (ABS) 1997, 'National Nutrition Survey: Selected Highlights', Australia, 1995, cat. No. 4802.0, ABS, Canberra.

Australian Bureau of Statistics (ABS) 2013, 'Profile of Health, Australia', Australia, 2011-13, cat. No. 4338.0, ABS, Canberra.

Barabási, AL, & Oltvai, ZN 2004, 'Network biology: understanding the cell's functional organization', *Nature Reviews Genetics*, vol. 5, no. 2, pp. 101-113.

Barker, DJ, Hales, CN, Fall, CH, Osmond, C, Phipps, K & Clark, PM 1993, 'Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth', *Diabetologia*, vol. 36, no. 1, pp. 62-67.

Barrès, R, Osler, ME, Yan, J, Fritz, T, Caidahl, K, Krook, A & Zierath, JR 2009, 'Non-CpG methylation of the PGC-1alpha promoter through DNMT3B controls mitochondrial density', *Cell Metabolism*, vol. 10, no. 3, pp. 189-198.

Barrès, R, Yan, J, Egan, B, Treebak, JT, Rasmussen, M, Fritz, T, Caidahl, K, Krook, A, O'Gorman, DJ & Zierath, JR 2012, 'Acute exercise remodels promoter methylation in human skeletal muscles', *Cell Metabolism*, vol. 15, no. 3, pp. 405-411.

Bauer, F, Elbers, CC, Adan, RA, Loos, RJ, Onland-Moret, NC, Grobbee, DE, van Vliet-Ostaptchouk, JV, Wijmenga, C & van der Schouw, YT 2009, 'Obesity genes identified in genome-wide association studies are associated with adiposity measures and potentially with nutrient-specific food preference', *The American Journal of Clinical Nutrition*, vol. 90, no. 4, pp. 951-959.

Bell, CG, Finer, S, Lindgren, CM, Wilson, GA, Rakyan, VK, Teschendorff, AE, Akan, P, Stupka, E, Down, TA, Prokopenko, I, Morison, IM, Mill, J, Pidsley, R, International Type 2 Diabetes 1q Consortium, Deloukas, P, Frayling, TM, Hattersley, AT, McCarthy, MI, Beck, S & Hitman, GA 2010, 'Integrated genetic and epigenetic analysis identifies haplotype-specific methylation in the FTO type 2 diabetes and obesity susceptibility locus', *PLoS One*, vol. 5, no. 11, e14040.

Bellou, E, Magkos, F, Kouka, T, Bouchalaki, E, Sklaveniti, D, Maraki, M, Tsekouras,YE, Panagiotakos, DB, Kavouras, SA, & Sidossis, LS 2013, 'Effect of high-intensity interval exercise on basal triglyceride metabolism in non-obese men', *Applied Physiology, Nutrition and Metabolism*, vol. 38, no. 8, pp. 823-329.

Berentzen, T, Kring SI, Holst, C, Zimmermann, E, Jess, T, Hansen, T, Pedersen, O, Toubro, S, Astrup, A & Sørensen, TIA 2008, 'Lack of association of fatness-related FTO gene variants with energy expenditure or physical activity', *The Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 7, pp. 2904-2908.

Berg, JM, Tymoczko, JL & Stryer, L 2002, 'Biochemistry: 5th Edition', New York: W H Freeman, section 16.2.3.

Bergström, J 1962, 'Muscle electrolytes in man', *Scandinavian Journal of Clinical & Laboratory Investigation*, vol. 14, pp. 511-513.

Berhane, F, Fite, A, Daboul, N, Al-Janabi, W, Msallaty, Z, Caruso, M, Lewis, MK, Yi, Z, Diamond, MP, Abou-Samra, AB & Seyoum, B 2015, 'Plasma lactate levels increase during hyperinsulinemic euglycemic clamp and oral glucose tolerance test', *Journal of Diabetes Research*, vol. 2015, e102054.

Berulava, T & Horsthemke, B 2010 'The obesity-associated SNPs in intro 1 of the FTO gene affect primary transcript levels', *European Journal of Human Genetics*, vol. 18, no. 9, pp. 1054-1056.

Berulava, T, Ziehe, M, Klein-Hitpass, L, Mladenov, E, Thomale, J, Rüther, U & Horsthemke, B 2013, 'FTO levels affect RNA modification and the transcriptome', *European Journal of Human Genetics*, vol. 21, no. 3, pp. 317-323.

Bielinski, R, Schutz, Y, & Jéquier, E 1985, 'Energy metabolism during the postexercise recovery in man', *The American Journal of Clinical Nutrition*, vol. 42, no. 1, pp. 69-82.

Bier, DM & Young, VR 1983, 'Exercise and blood pressure: nutritional considerations', *Annals of Internal Medicine*, vol. 98, no. 5 (Pt 2), pp. 864-869.

Bird, A 2007, 'Perceptions of epigenetics', *Nature*, vol. 447, no. 7143, pp. 396-398.

Boissel, S, Reish, O, Proulx, K, Kawagoe-Takaki, H, Sedgwick, B, Yeo, GSH, Meyre, D, Golzio, C, Molinari, F, Kadhorm, N, Etchevers, HC, Saudek, V, Farooqi, IS, Froguel, P, Lindahl, T, O'Rahilly, S, Munnich, A & Colleaux, L 2009, 'Loss-of-function mutation in the dioxygenase-encoding FTO gene causes severe growth retardation and multiple malformations', *American Journal of Human Genetics*, vol. 85, no. 1, pp. 106-111.

Bonnard, C, Durand, A, Peyrol, S, Chanseaume, E, Chauvin, MA, Morio, B, Vidal, H & Rieusset1, J 2008, 'Mitochondrial dysfunction results from oxidative stress in the skeletal muscle of diet-induced insulin-resistant mice', *The Journal of Clinical Investigations*, vol. 118, no. 2, pp. 789–800.

Booth, FW, & Watson, PA 1985, 'Control of adaptations in protein levels in response to exercise', *Federation Proceedings*, vol. 44, no. 7, pp. 2292-2300.

Borg, G 1998, 'Borg's perceived exertion and pain scale', Champaign, IL, US: Human Kinetics.

Boughton, BA, Callahan, DL, Silva, C, Bowne, J, Nahid, A, Rupasinghe, T, Tull DL, McConville, MJ, Bacic, A, & Roessner, U 2011, 'Comprehensive profiling and quantitation of amino group containing metabolites', *Analytical Chemistry*, vol. 83, no. 19, pp. 7523-7530.

Bouwman, FG, Boer, JMA, Imholz, S, Wang, P, Verschuren, WMM, Dollé, MET & Mariman, ECM 2014, 'Gender-specific genetic associations of polymorphisms in ACE, AKR1C2, FTO and MMP2 with weight gain over a 10-year period', *Genes and Nutrition*, vol. 9, no. 6, pp. 434.

Bravard, A, Lefai, E, Meugnier, E, Pesenti, S, Disse, E, Vouillarmet, J, Peretti, N, Rabasa-Lhoret, R, Laville, M, Vidal, H & Rieusset, J 2011, 'FTO is increased in muscle during type 2 diabetes, and its overexpression in myotubes alters insulin signaling, enhances lipogenesis and ROS production, and induces mitochondrial dysfunction', *Diabetes*, vol. 60, no. 1, pp. 258-268.

Bravard, A, Vial, G, Chauvin, MA, Rouille, Y, Bailleul, B, Vidal, H & Rieusset, J 2014, 'FTO contributes to hepatic metabolism regulation through regulation of leptin action and STAT3 signalling in liver', *Cell Communication and Signal*, vol. 12, no. 4, pp. 1-13.

Brugnara, L, Vinaixa, M, Murillo, S, Samino, S, Rodriguez, MA, Beltran, A, Lerin, C, Davison, G, Correig, X, & Novials, A 2012, 'Metabolomics approach for analysing the effects of exercise in subjects with type 1 diabetes mellitus', *PLoS One*, vol. 7, no. 7, e40600.

Brunkwall, L, Ericson, U, Hellstrand, S, Gullberg, B, Orho-Melander, M & Sonestedt, E 2013, 'Genetic variation in the fat mass and obesity-associated gene (FTO) in association with food preferences in healthy adults', *Food and Nutrition Research*, vol. 57, no. 20028.

Bylesjö, M, Eriksson, D, Kusano, M, Moritz, T & Trygg, J 2007, 'Data integration in plant biology: the O2PLS method for combined modeling of transcript and metabolite data', *The Plant Journal: for cell and molecular biology*, vol. 52, no. 6, pp. 1181-1191.

Calles, J, Cunningham, JJ, Nelson, L, Brown, N, Nadel, E, Sherwin, RS & Felig, P 1983, 'Glucose turnover during recovery from intensive exercise', *Diabetes*, vol. 32, no. 8, pp. 734-738.

Carone, BR, Fauquier, L, Habib, N, Shea, JM, Hart, CE, Li, R, Bock, C, Li, C, Zamore, PD Meissner, A, Weng, Z, Hofmann, HA, Friedman, N & Rando1, OJ 2010, 'Paternally-induced transgenerational environmental reprogramming of metabolic gene expression in mammals', *Cell,* vol. 143, no. 7, pp. 1084–1096.

Cecil, JE, Tavendale, R, Watt, P, Hetherington, MM & Palmer, CND 2008, 'An obesity-associated FTO gene variant and increased energy intake in children', *The New England Journal of Medicine*, vol. 359, no. 1, pp. 2558-2566.

Charron, MJ, Brosius, FC 3rd, Alper, SL & Lodish, HF 1989, 'A glucose transport protein expressed predominately in insulin-responsive tissues', *Proceedings of the National Academy of Sciences of the USA*, vol. 86 no. 8, pp. 2535-2539.

Chen, T, Hao, YJ, Zhang, Y, Li, MM, Wang, M, Han, W, Wu, Y, Lv, Y, Hao, J, Wang, L, Li, A, Yang, Y, Jin, KX, Zhao, X, Li, Y, Ping, XL, Lai, WY, Wu, LG, Jiang, G, Wang, HL, Sang, L, Wang, XJ, Yang, YG & Zhou, Q 2015, 'm^6A RNA methylation is regulated by microRNAs and promotes reprogramming to pluripotency', *Cell Stem Cell*, vol. 16, no. 3, pp. 289-301.

Cheung, MK, Gulati, P, O'Rahilly, S & Yeo, GSH 2013, 'FTO expression is regulated by availability of essential amino acids', *International Journal of Obesity (London)*, vol. 37, no. 5, pp. 744-747.

Chorell, E, Moritz, T, Branth, S, Antti, H & Svensson, MB 2009, 'Predictive metabolomics evaluation of nutrition-modulated metabolic stress responses in human blood serum during the early recovery phase of strenuous physical exercise', *Journal of Proteome Research*, vol. 8, no. 6, pp. 2966-2977.

Church, C, Lee, S, Bagg, EAL, McTaggart, JS, Deacon, R, Gerken, T, Lee, A, Moir, L, Mecinovic, J, Quwailid, MM, Schofield, CJ, Ashcroft, FM & Cox, RD 2009, 'A mouse model for the metabolic effects of the human fat mass and obesity associated FTO gene', *PLoS Genetics*, vol. 5, no. 8, e1000599.

Church, C, Moir, L, McMurray, F, Girard, C, Banks, GT, Teboul, L, Wells, S, Bruning, JC, Nolan, PM, Ashcroft, FM & Cox, RD 2010, 'Overexpression of Fto leads to increased food intake and results in obesity', *Nature Genetics*, vol. 42, no. 12, pp. 1086-1092.

Claussnitzer, M, Dankel, SN, Kim, K, Quon, G, Meuleman, W, Haugen, C, Glunk, V, Sousa, IS, Beaudry, JL, Puviindran, V, Abdennur, NA, Liu, J, Svensson, P-A, Hsu, Y-H, Drucker, DJ, Mellgren, G, Hui, C, Hauner, H & Kellis, M 2015, 'FTO obesity variant circuitry and adipocyte browning in humans', *The New England Journal of Medicine*, vol. 373, no. 2, pp. 895-907.

Coffey, VG & Hawley, JA 2007, 'The molecular bases of training adaptation', *Sports Medicine*, vol. 37, no. 9, pp. 737-763.

Colagiuri, S, Lee, CMY, Colagiuri, R, Magliano, D, Shaw, JE, Zimmet, PZ & Caterson ID 2010, 'The cost of overweight and obesity in Australia', *The Medical Journal of Australia*, vol. 192, no. 5, pp. 260-264.

Compher, C, Frankerfield, D, Keim, N & Roth-Yousey, L 2006, 'Best practice methods to apply to measurement of resting metabolic rates in adults: a systematic review', *Journal of the American Dietetic Association*, vol. 106, no. 6, pp. 881-903.

Corpeleijn, E, Mensink, M, Kooi, ME, Roekaerts, PM, Saris, WH & Blaak, EE 2008, 'Impaired skeletal muscle substrate oxidation in glucose-intolerant men improves after weight loss', *Obesity*, vol.16, no. 5, pp.1025-1032.

Corpeleijn, E, Petersen, L, Holst, C, Saris, WH, Astrup, A, Langin, D, MacDonald, I, Martinez, JA, Oppert, J, Polak, J, Pedersen, O, Froguel, P, Arner, P, Sørensen, TIA & Blaak, EE 2010, 'Obesity-related polymorphisms and their associations with the ability to regulate fat oxidation in obese Europeans: the NUGENOB study', *Obesity*, vol. 18, no. 7, pp. 1369-1377.

Cummings, DE & Overduin, J 2007, 'Gastrointestinal regulation of food intake', *The Journal of Clinical Investigations*, vol. 117, no. 1, pp. 13-23.

Curradi, M, Izzo, A, Badaracco, G & Landsberger, N 2002, 'Molecular mechanisms of gene silencing mediated by DNA methylation', *Molecular and Cellular Biology*, vol. 22, no. 9, pp. 3157–3173.

Danaher, J, Gerber, T, Wellard, RM, Stathis, CG, & Cooke, MB 2016, 'The use of metabolomics to monitor simultaneous changes in metabolic variables following supramaximal low volume high intensity exercise', *Metabolomics*, vol. 12, no. 7, pp. 1-13.

Davenport, JR, Watts, AJ, Roper, VC, Croyle, MJ, van Groen, T, Wyss, JM, Nagy, TR, Kesterson, RA & Yoder, BK 2007, 'Disruption of intraflagellar transport in adult mice leads to obesity and slow-onset cystic kidney disease', *Current Biology*, vol. 17, no. 18, pp. 1586–1594

Desrosiers, R, Friderici, K & Rottman, F 1974, 'Identification of methylated nucleosides in messenger RNA from Novikoff hepatoma cells', *Proceedings of the National Academy of Sciences of the USA*, vol. 71, no. 10, pp. 3971-3975.

Dina, C, Meyre, D, Gallina, S, Durand, E, Korner, A, Jacobson, P, Carlsson, LM, Kiess, W, Vatin, V, Lecoeur, C, Delplangue, J, Vaillant, E, Pattou, F, Ruiz, J, Weill, J, Levy-Marchal, C, Horber, F, Potoczna, N, Hercberg, S, Le Stunff, C, Bougnères, P, Kovacs, P, Marre, M, Balkau, B, Cauchi, S, Chèvre, JC & Froguel, P 2007, 'Variation in FTO contributes to childhood obesity and severe adult obesity', *Nature Genetics*, vol. 39, no. 6, pp. 724-726.

Dominissini, D, Moshitch-Moshkovitz, S, Schwartz, S, Salmon-Divon, M, Ungar, L, Osenberg, S, Cesarkas, K, Jacob-Hirsch, J, Amariglio, N, Kupiec, M, Sorek, R & Rechavi, G 2012, 'Topology of the human and mouse m^6A RNA methylomes revealed by m^6A-seq', *Nature*, vol. 485, no. 7397, pp. 201-206.

Dunn, WB, Broadhurst, DI, Atherton, HJ, Goodacre, R & Griffin, JL 2011, 'Systems level studies of mammalian metabolomes: the roles of mass spectrometry and nuclear magnetic resonance spectroscopy', *Chemical Society Reviews*, vol. 40, no. 1, pp. 387-426.

Egan, B, Carson, BP, Garcia-Roves, PM, Chibalin, AV, Sarsfield, FM, Barron, N, McCaffrey, N, Moyna, NM, Zierath, JR & O'Gorman, DJ 2010, 'Exercise intensity-dependent regulation of peroxisome proliferator-activated receptor coactivator-1 mRNA abundance is associated with differential activation of upstream signalling kinases in human skeletal muscle', *The Journal of Physiology*, vol. 15, no. 588 (Pt 10), pp. 1779-1790.

Egan, B, O'Connor, PL, Zierath, JR & O'Gorman, DJ 2013 'Time course analysis reveals gene-specific transcript and protein kinetics of adaptation to short-term aerobic exercise training in human skeletal muscle', *PLoS One*, vol. 8, no. 9, e74098.

Egger, G, Liang, G, Aparicio, A & Jones, PA 2004, 'Epigenetics in human disease and prospects for epigenetic therapy', *Nature*, vol. 27, no. 6990, pp. 457-463.

Eriksson, L, Johansson, E, Kettaneh-Wold, N, Trygg, J, Wikström, C & Wold, S 2006, 'Multi- and megavariant data analysis: Part I basic principles and applications', Umetrics Academy, Umeå.

Evans, WS, Phinney, S & Young, V 1982, 'Suction applied to a muscle biopsy maximised sample size', *Medicine and Science in Sports and Exercise*, vol. 14, no. 1, pp. 101-102.

Ferraro, E, Giammarioli, AM, Chiandotto, S, Spoletini, I & Rosano, G 2014, 'Exercise-induced skeletal muscle remodeling and metabolic adaptation: redox signaling and role of autophagy', *Antioxidants and Redox Signaling*, vol. 21, no. 1, pp. 154-167.

Fischer, J, Koch, L, Emmerling, C, Vierkotten, J, Peters, T, Brüning, JC & Rüther, U 2009, 'Inactivation of the Fto gene protects from obesity', *Nature*, vol. 458, no. 7240, pp. 894-898.

Flatt, JP 1988, 'Importance of nutrient balance in body weight regulation', *Diabetes Metabolism Reviews*, vol. 4, no. 6, pp. 571-581.

Fraga, MF, Ballestar, E, Paz, MF, Ropero, S, Setien, F, Ballestar, ML, Heine-Suñer, D, Ciqudosa, JC, Urioste, M, Benitez, J, Boix-Chornet, M, Sanchez-Aguilera, A, Ling, C, Carlsson, E, Poulsen, P, Vaag, A, Stephan, Z, Spector, TD, Wu, YZ, Plass, C & Esteller, M 2005, 'Epigenetic differences arise during the lifetime of monozygotic twins', *Proceedings of the National Academy of Sciences of the USA*, vol. 102, no. 30, pp. 10604-10609.

Frayling, TM, Timpson, NJ, Weedon, MN, Zeggini, E, Freathy, RM, Lindgren, CM, Perry, JR, Elliott, KS, Lango, H, Rayner, NW, Shields, B, Harries, LW, Barrett, JC, Ellard, S, Groves, CJ, Knight, B, Patch, AM, Ness, AR, Ebrahim, S, Lawlor, DA, Ring, SM, Ben-Shlomo, Y, Jarvelin, MR, Sovio, U, Bennett, AJ, Melzer, D, Ferrucci, L, Loos, RJ, Barroso, I, Wareham, NJ, Karpe, F, Owen, KR, Cardon, LR, Walker, M, Hitman, GA, Palmer, CN, Doney, AS, Morris, AD, Smith, GD, Hattersley, AT & McCarthy MI 2007, 'A common variant in the FTO gene is associated with body mass index and predisposes to childhood and adult obesity', *Science*, vol. 316, no. 5826, pp. 889-894.

Fredriksson, R, Hägglund, M, Olszewski, PK, Stephansson, O, Jacobsson, JA, Olszewska, AM, Levine, AS, Lindbolm, J & Schiöth, HB 2008, 'The obesity gene, FTO, is of ancient origin, up-regulated during food deprivation and expressed in neurons of feeding-related nuclei of the brain', *Endocrinology*, vol. 149, no. 5, pp. 2062-2071.

Friedlander, AL, Casazza, GA, Horning, MA, Buddinger, TF, & Brooks, GA 1998, 'Effects of exercise intensity and training on lipid metabolism in young women', *American Journal of Physiology*, vol. 275, no. 5, E853-863.

Fu, Y, Dominissini, D, Rechavi, G & He, C 2014, 'Gene expression regulation mediated through reversible m^6A RNA methylation', *Nature Review Genetics*, vol. 15, no. 5, pp. 292-306.

Galgani, J & Ravussin, E 2008, 'Energy metabolism, fuel selection and body weight regulation', *The International Journal of Obesity (London)*, vol. 32, no. 7, S109-S119.

Galgani, JE, Heilbronn, LK, Azuma, K, Kelley, DE, Albu, JB, Pi-Sunyer, X, Smith, SR, Ravussin, E & Look AHEAD Adipose Research Group 2008*a*, 'Metabolic flexibility in response to glucose is not impaired in people with type 2 diabetes after controlling for glucose disposal rate', *Diabetes*, vol. 57, no. 4, pp. 841-845.

Galgani, JE, Moro, C & Ravussin, E 2008*b*, 'Metabolic flexibility and insulin resistance', *American Journal of Physiology, Endocrinology and Metabolism*, vol. 295, no. 5, E1009-E1017.

Gao, X, Shin, YH, Li, M, Wang, F, Tong, Q & Zhang, P 2010, 'The fat mass and obesity associated gene FTO functions in the brain to regulate postnatal growth in mice', *PLoS One*, vol. 5, no. 11, e14005.

Gerber, T, Borg, ML, Hayes, A, & Stathis, CG 2014, 'High-intensity intermittent cycling increases purine loss compared with workload-matched continuous moderate intensity cycling', *European Journal of Applied Physiology*, vol. 114, no. 7, pp. 1513-1520.

Gerken, T, Girard, CA, Tung, YCL, Webby, CJ, Saudek, V, Hewitson, KS, Yeo, GSH, McDonough, MA, Cunliffe, S, McNeill, LA, Galvanovskis, J, Rorsman, P, Robins, P, Prieur, X, Coll, AP, Ma, M, Jovanovic, Z, Farooqi, IS, Sedgwick, B, Barroso, I, Lindahl, T, Ponting, CP, Ashcroft, FM, O'Rahilly, S & Scholfield, CJ 2007, 'The obesity-associated FTO gene encodes a 2-oxoglutarate-dependent nucleic acid demethylase', *Science*, vol. 318, no. 5855, pp. 1469-1472.

Gibala, MJ, Little, JP, MacDonald, MJ, & Hawley, JA 2012, 'Physiological adaptations to low-volume, high-intensity interval training in health and disease', *The Journal of Physiology*, vol. 590, no. 5, pp. 1077-1084.

Gibala, MJ, Little, JP, van Essen, M, Wilkin, GP, Burgomaster, KA, Safdar, A, Raha, S, & Tarnopolsky, MA 2006, 'Short-term sprint interval versus traditional endurance training: similar initial adaptations in human skeletal muscle and exercise performance', *The Journal of Physiology*, vol. 575, no. 3, pp. 901-911.

Gibala, MJ, MacLean, DA, Graham, TE & Saltin, B 1997*a*, 'Anaplerotic processes in human skeletal muscle during brief dynamic exercise', *The Journal of Physiology*, vol. 502 (Pt 3), pp. 703-713.

Gibala, MJ, McGee, SL, Garnham, AP, Howlett, KF, Snow, RJ & Hargreaves, M 2009, 'Brief intense interval exercise activates AMPK and p38 MAPK signaling and increases the expression of PGC-1α in human skeletal muscle', *Journal of Applied Physiology*, vol. 106 no. 3, pp. 929-934.

Gibala, MJ, Tarnopolsky, MA & Graham, TE 1997*b*, Tricarboxylic acid cycle intermediates in human muscle at rest and during prolonged cycling', *The American Journal of Physiology*, vol. 272, no. 2 (Pt 1), E239-244.

Gieger, C, Geistlinger, L, Altmaier, E, de Angelis, MH, Kronenberg, F, Meitinger, T, Mewes, HW, Wichmann, HE, Weinberger, KM, Adamski, J Illig, T & Suhre, K 2008, 'Genetics meets metabolomics: A genome-wide association study of metabolite profiles in human serum', *PLoS Genetics*, vol. 4, no. 11, e1000282.

Grootveld, M 2012, 'Introduction to the applications of chemometric techniques in 'omics' research: common pitfalls, misconceptions and 'rights and wrongs', *Metabolic Profiling: Disease and Xenobiotics, Royal Society of Chemistry*, pp. 1-34.

Grunnet, LG, Brøns, C, Jacobsen, S, Nilsson, E, Astrup, A, Hansen, T, Pedersen, O, Poulsen, P, Quistorff, B & Vaag, A 2009*a*, 'Increased recovery rates of phosphocreatine and inorganic phosphate after isometric contraction in oxidative muscle fibers and elevated hepatic insulin resistance in homozygous carriers of the A-allele of FTO rs9939309', *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 2, pp. 596-602.

Grunnet, LG, Nilsson, E, Ling, C, Hansen, T, Pedersen, O, Groop, L, Vaag, A & Poulsen, P 2009*b*, 'Regulation and function of FTO mRNA expression in human skeletal muscle and subcutaneous adipose tissue', *Diabetes*, vol. 58, no. 10, pp. 2402-2408.

Gulati, P, Avezov, E, Ma, M, Antrobus, R, Lehner, P, O'Rahilly, S & Yeo, GSH 2014, 'Fat mass and obesity-related (FTO) shuttles between the nucleus and cytoplasm', *Bioscience Reports*, vol. 34, no. 5, e00144.

Gulati, P, Cheung, MK, Antrobus, R, Church, CD, Harding, HP, Tung, YCL, Rimmington, D, Ma, M, Ron, D, Lehner, PJ, Ashcroft, FM, Cox, RD, Coll, AP, O'Rahilly, S & Yeo, GSH 2013, 'Role for the obesity-related FTO gene in the cellular sensing of amino acids', *Proceedings of the National Academy of Sciences of the U.S.A*, vol. 110, no. 7, pp. 2557-2562.

Gumbiner, B, Thorburn, AW, Ditzler, TM, Bulacan, F & Henry, RR 1992, 'Role of impaired intracellular glucose metabolism in the insulin resistance of aging', *Metabolism*, vol. 41, no. 10, pp. 1115-1121.

Guo, F, Zhang, Y, Zhang, C, Wang, S, Ni, Y & Zhao, R 2015, 'Fat mass and obesity associated (FTO) gene regulated gluconeogenesis in chicken embryo fibroblast cells', *Comparative Biochemistry and Physiology, Part A Molecular and Integrative Physiology*, vol. 179, pp. 149-156.

Hainer, V, Stunkard, AJ, Kunesová, M, Parízková, J, Stich, V & Allison, DB 2000, 'Intrapair resemblance in very low calorie diet-induced weight loss in female obese identical twins', *International Journal of Obesity and Related Metabolic Disorders*, vol. 24, no. 8, pp. 1051-1057.

Hakanen, M, Raitakari, OT, Lehtimäki, T, Peltonen, N, Pahkala, K, Sillanmäki, L, Lagström, H, Viikari, J, Simell, O & Rönnemaa, T 2009, 'FTO genotype is associated with body mass index after the age of seven years but not with energy intake of leisure-time physical activity', *The Journal of Clinical Endocrinology and Metabolism*, vol. 94, no. 4, pp. 1281-1287.

Hall, KD, Heymsfield, SB, Kemnitz, JW, Klein, S, Schoeller, DA & Speakman, JR 2012, 'Energy balance and its components: implications for body weight regulation', *The American Journal of Clinical Nutrition*, vol. 95, no. 4, pp. 989–994.

Han, Z, Niu, T, Chang, J, Lei, X, Zhao, M, Wang, Q, Cheng, W, Wang, J, Feng, Y & Chai 2010, 'Crystal structure of the FTO protein reveals basis for its substrate specificity', *Nature*, vol. 464, no. 7292, pp. 1205-1209.

Hardy, R, Wills, AK, Wong, A, Elks, CE, Wareham, NJ, Loos, RJ, Kuh, D & Ong, KK 2010, 'Life course variations in the association between FTO and MC4R gene variants and body size', *Human Molecular Genetics*, vol. 19, no. 3, pp. 545-552.

Haupt, A, Thamer, C, Machann, J, Kirchhoff, K, Stefan, N, Tschritter, O, Machicao1, F, Schick, F, Häring1, H & Fritschel, A 2008, 'Impact of variation in the FTO gene on whole body fat distribution, ectopic fat, and weight loss', *Obesity*, vol. 16, no. 8, pp. 1969-1972.

Heijmans, BT, Kremer, D, Tobi, EW, Boomsma, DI & Slagboom, PE 2007, 'Heritable rather than age-related environmental and stochastic factors dominate variation in DNA methylation of the human IGF2/H19 locus', *Human Molecular Genetics*, vol. 16, no. 5, pp. 547-554.

Hess, ME & Brüning, JC 2014, 'The fat mass and obesity-associated (FTO) gene: obesity and beyond?', *Biochimica et Biophysica Acta*, vol. 1842, no. 10, pp. 2039-2047.

Hess, ME, Hess, S, Meyer, KD, Verhagen, LA, Koch, L, Brönneke, HS, Dietrich, MO, Jordan, SD, Saletore, Y, Elemento, O, Belgardt, BF, Franz, T, Horvath, TL, Rüther, U, Jaffrey, SR, Kloppenburg, P & Brüning, JC 2013, 'The fat mass and obesity associated gene (Fto) regulates activity of the dopaminergic midbrain circuitry', *Nature Neuroscience*, vol. 16, no. 8, pp. 1042-1048.

Hill, JO, Peters, JC & Wyatt, HR 2009, 'Using the energy gap to address obesity: A commentary', *Journal of the American Dietetic Association*, vol. 109, no. 11, pp. 1848-1853.

Ho, JE, Larson, MG, Vasan, RS, Ghorbani, A, Cheng, S, Rhee, EP, Florez, JC, Clish, CB, Gerszten, RE & Wang, TJ 2013, 'Metabolite profiles during oral glucose challenge', *Diabetes*, vol. 62, no. 8, pp. 2689-2698.

Hood, MS, Little, JP, Tarnopolsky, MA, Myslik, F & Gibala, MJ 2011, 'Low-volume interval training improves muscle oxidative capacity in sedentary adults', *Medicine and Science in Sports and Exercise*, vol. 43, no. 10, pp. 1849-1856.

Horton, TJ, Drougas, H, Brachey, A, Reed, GW, Peters, JC & Hill, JO 1995, 'Fat and carbohydrate overfeeding in humans: different effects on energy storage', *The American Journal of Clinical Nutrition*, vol. 62, no. 1, pp. 19-29.

Hubacek, JA, Pitha, J, Adamkova, V, Lanska, V & Poledne, R 2009, 'A common variant in the FTO gene is associated with body mass index in males and postmenopausal females but not in premenopausal females. Czech post-MONICA and 3PMFs studies', *Clinical Chemistry and Laboratory Medicine*, vol. 47, no. 4, pp. 397-390.

Jacobsson, JA, Almén, MS, Benedict, C, Hedberg, LA, Michaëlsson, K, Brooks, S, Kullberg, J, Axelsson, T Johansson, L, Ahlström, H, Fredriksson, R, Lind, L, & Schiöth, HB 2011, 'Detailed analysis of variants in FTO in association with body composition in a cohort of 70-year-olds suggests a weakened effect among elderly', *PLoS One*, vol. 6, no. 5, e20158.

Jacobsson, JA, Danielsson, P, Svensson, V, Klovins, J, Gyllensten, U, Marcus, C, Schiöth, HB & Fredriksson, R 2008, 'Major gender difference in association of FTO gene variant among severely obese children with obesity and obesity related phenotypes', *Biochemical and Biophysical Research Communications*, vol. 368, no. 3, pp. 476-482.

Jacobsson, JA, Schiöth, HB & Fredriksson, R 2012, 'The impact of intronic single nucleotide polymorphisms and ethnic diversity for studies on the obesity gene FTO', *Obesity Reviews*, vol. 13, no. 12, 1096-1109.

Jain-Ghai, S, Cameron, JM, A, Maawali, A, Blaser, S, MacKay, N, Robinson, B & Raiman, J 2013, 'Complex II deficiency--a case report and review of the literature', *American Journal of Medical Genetics*, vol. 161A, no. 2, pp. 285-294.

Jeukendrup, AE & Wallis, GA 2005 'Measurement of substrate oxidation during exercise by means of gas exchange measurements', *International Journal of Sports Medicine*, vol. 26, no. 1, pp. 28-37.

Jia, G, Fu, Y & He, C 2013, 'Reversible RNA adenosine methylation in biological regulation', *Trends in Genetics*, vol. 29, no. 2, pp. 108-115.

Jia, G, Fu, Y, Zhao, X, Dai, Q, Zheng, G, Yang, Y, Yi, C, Lindahl, T, Pan, Y, Yang, YG & He, C 2011, 'N^6-methyladenosine in nuclear RNA is a major substrate of the obesity associated FTO', *Nature Chemical Biology*, vol. 7, no. 12, pp. 885-887.

Jia, G, Yang, CG, Yang, S, Jian, X, Yi, C, Zhou, Z & Hea, C 2008, 'Oxidative demethylation of 3-methylthymine and 3-methyluracil in single-stranded DNA and RNA by mouse and human FTO', *FEBS Letters*, vol. 582 no. 23-24, pp. 3313-3319.

Jia, X, Nie, Q, Lamont, SJ & Zhang, X 2012, 'Variation in sequence and expression of the avian FTO, and association with glucose metabolism, body weight, fatness and body composition in chicken', *International Journal of Obesity*, vol. 36, no. 8, pp. 1054-1061.

Jiao, Y, Zhang, J, Lu, L, Xu, J & Qin, L 2016, 'The Fto gene regulates the proliferation and differentiation of pre-adipocytes in vitro', *Nutrients*, vol. 8, no. 2, pp. 102.

Jonsson, A, Renström, F, Lyssenko, V, Brito, EC, Isomaa, B, Berglund, G, Nilsson, PM, Groop, L & Franks, PW 2009, 'Assessing the effect of interaction between an FTO variant (rs9939609) and physical activity on obesity in 15,925 Swedish and 2,511 Finnish adults', *Diabetologia*, vol. 57, no. 7, pp. 1334-1338.

Kaiyala, KJ & Schwartz, MW 2011, 'Toward a more complete (and less controversial) understanding of energy expenditure and its role in obesity pathogenesis', *Diabetes*, vol. 60, no. 1, pp. 17-23.

Kanehisa, M, & Goto, S 2000, 'KEGG: Kyoto encyclopedia of genes and genomes', *Nucleic Acids Research*, vol. 28, no. 1, pp. 27-30.

Karra, E, O'Daly, OG, Choudhury, AI, Yousseif, A, Millership, S, Neary, MT, Scott, WR, Chandarana, K, Manning, S, Hess, ME, Iwakura, H, Akamizu, T, Millet, Q, Gelegen, C, Drew, ME, Rahman, S, Emmanuel, JJ, Williams, SC, Rüther, UU, Brüning, JC, Withers, DJ, Zelaya, FO & Batterham, RL 2013, 'A link between FTO, ghrelin, and impaired brain food-cue responsivity', *The Journal of Clinical Investigation*, vol. 123, no. 8, pp. 3539-3551.

Kathiresan, S, Melander, O, Guiducci, C, Surti, A, Burtt, NP, Rieder, MJ, Cooper, GM, Roos, C, Voight, BF, Havulinna, AS, Wahlstrand, B, Hedner, T, Corella, D, Tai, ES, Ordovas, JM, Berglund, G, Vartiainen, E, Jousilahti, P, Hedblad, B, Taskinen, MR, Newton-Cheh, C, Salomaa, V, Peltonen, L, Groop, L, Altshuler, DM & Orho-Melander, M 2008, 'Six new loci associated with blood low-density lipoprotein cholesterol, high-density lipoprotein cholesterol or triglycerides in humans', *Nature Genetics*, vol. 40, no. 2, pp. 189-197.

Katz, A, Broberg, S, Sahlin, K & Wahren, J 1986, 'Leg glucose uptake during maximal dynamic exercise in humans', *The American Journal of Physiology*, vol. 251, no. 1 (Pt 1), E65-70.

Kelley, DE 2005, 'Skeletal muscle fat oxidation: timing and flexibility are everything', *The Journal of Clinical Investigation*, vol. 115, no. 7, pp. 1699-1702.

Kelley, DE, Goodpaster, B, Wing, RR & Simoneau, JA 1999, 'Skeletal muscle fatty acid metabolism in association with insulin resistance, obesity and weight loss', *The American Journal of Physiology*, vol. 277, no. 1, E1130-1141.

Kelley, D, Mokan, M & Veneman, T 1994, 'Impaired postprandial glucose utilization in non-insulin-dependent diabetes mellitus', *Metabolism*, vol. 43, no. 12, pp. 1549-1557.

Kelley, DE & Simoneau, JA 1994, 'Impaired free fatty acid utilization by skeletal muscle in non-insulin-dependent diabetes mellitus', *The Journal of Clinical Investigation*, vol. 94, no. 6, pp. 2349-2356.

Kiens, B, & Richter, RA 1998, 'Utilization of skeletal muscle triacylglycerol during postexercise recovery in humans', *American Journal of Physiology, Endocrinology and Metabolism*, vol. 275, no. 1, pp. 332-337.

Kilpeläinen, TO, Qi, L, Brage, S, Sharp, SJ, Sonestedt, E, Demerath, E, Ahmad, T, Mora, S, Kaakinen, M, Sandholt, CH, Holzapfel, C, Autenrieth, CS, Hyppönen, E, Cauchi, S, He, M, Kutalik, Z, Kumari, M, Stančáková, A, Meidtner, K, Balkau, B, Tan, JT, Mangino, M, Timpson, NJ, Song, Y, Zillikens, MC, Jablonski, KA, Garcia, ME, Johansson, S, Bragg-Gresham, JL, Wu, Y, van Vliet-Ostaptchouk, JV, Onland-Moret, NC, Zimmermann, E, Rivera, NV, Tanaka, T, Stringham, HM, Silbernage, G, Kanoni, S, Feitosa, MF, Snitker, S, Ruiz, JR, Metter, J, Martinez Larrad, MT, Atalay, M, Hakanen, M, Amin, N, Cavalcanti-Proença, C, Grøntved, A, Hallmans, G, Jansson, J, Kuusisto, J, Kähönen, M, Lutsey, PL, Nolan , JJ, Palla, L, Pedersen, O, Pérusse, L, Renström, F, Scott, RA, Shungin, D, Sovio, U, Tammelin, TH, Rönnemaa, T, Lakka, TA, Uusitupa, M, Rios, MS, Ferrucci, L, Bouchard, C, Meirhaeghe, A, Fu, M, Walker, M, Borecki, IB, Dedoussis, GV, Fritsche, A, Ohlsson, C, Boehnke, M, Bandinelli, S, van Duijn, CM, Ebrahim, S, Lawlor, DA, Gudnason, V, Harris, TB, Sørensen, TIA, Mohlke, KL, Hofman, A, Uitterlinden, AG, Tuomilehto, J, Lehtimäki, T, Raitakari, O, Isomaa, B, Njølstad, PR, Florez, JC, Liu, S, Ness, A, Spector, TD, Tai, ES, Froguell, P, Boeing, H, Laakso, M, Marmot, M, Bergmann, S, Power, C, Khaw, K, Chasman, D, Ridker, P, Hansen, T, Monda, KL, Illig, T, Järvelin, M, Wareham, NJ, Hu, FB, Groop, LC, Orho-Melander, M, Ekelund, U, Franks, PW & Loos, RJF 2011, 'Physical activity attenuates the influence of FTO variants on obesity risk: a meta-analysis of 218,166 adults and 19,268 children', *PLoS Medicine*, vol. 8, no. 11, e1001116.

Kimber, NE, Heigenhauser, GJF, Spriet, LL, & Dyck, DJ 2003, 'Skeletal muscle fat and carbohydrate metabolism during recovery from glycogen-depleting exercise in humans', *The Journal of Physiology*, vol. 548, no. 3, pp. 919-927.

Kirchner, H, Osler, ME, Krook, A & Zierath, JR 2013, 'Epigenetic flexibility in metabolic regulation: disease cause and prevention?', *Trends in Cell Biology*, vol. 23, no. 5, pp. 203-209.

Kjeldahl, K, Rasmussen, MA, Hasselbalch, AL, Kyvik, KO, Christiansen, L, Rezzi, S, Kochhar, S, Sørensen, TIA & Bro, R 2014, 'No genetic footprints of the fat mass and obesity associated (FTO) gene in human plasma ^{1}H CPMG NMR metabolic profiles', *Metabolomics*, vol. 10, no. 1, pp. 132-140.

Klose, RJ & Bird, AP 2006, 'Genomic DNA methylation: the mark and its mediators', *Trends in Biochemical Sciences*, vol. 31, no. 2, pp. 89-97.

Kring, SI, Holst, C, Zimmermann, E, Jess, T, Berentzen, T, Tounbro, S, Hansen, T, Astrup, A, Pedersen, O & Sørensen, TIA 2008, 'FTO gene associated fatness in relation to body fat distribution and metabolic traits throughout a broad range of fatness', *PLoS One*, vol. 3, no. 8, e2958.

Kulkarni, SS, Salehzadeh, F, Fritz, T, Zierath, JR, Krook, A & Osler, ME 2012, 'Mitochondrial regulators of fatty acid metabolism reflect metabolic dysfunction in type 2 diabetes mellitus', *Metabolism*, vol. 61, no. 2, pp. 175-185.

Kumar, R & Indrayan, A 2011, 'Receiver operating characteristic (ROC) curve for medical researchers', *Indian Pediatrics*, vol. 48, no. 4, pp. 277-287.

LaFramboise, T 2009, 'Single nucleotide polymorphism arrays: a decade of biological, computational and technological advances', *Nucleic Acid Research*, vol. 37, no. 13, pp. 4181-4193.

Lee, HJ, Kim, IK, Kang, JH, Ahn, Y, Han, BG, Lee, JY & Song, J 2010, 'Effects of common FTO gene variants associated with BMI on dietary intake and physical activity in Koreans', *Clinica Chimica Acta*, vol. 411, no. 21-22, pp. 1716-1722.

León-Mimila, P, Villamil-Ramírez, H, Villalobos-Comparán, M, Villarreal-Molina, T, Romero-Hidalgo, S, López-Contreras, B, Gutiérrez-Vidal, R, Vega-Badillo, J, Jacobo-Albavera, L, Posadas-Romeros, C, Canizalez-Román, A, Río-Navarro, BD, Campos-Pérez, F, Acuña-Alonzo, V, Aguilar-Salinas, C & Canizales-Quinteros, S 2013, 'Contribution of common genetic variants to obesity and obesity-related traits in mexican children and adults', *PLoS One*, vol. 8, no. 8, e70640.

Lewis, GD, Farrell, L, Wood, MJ, Martinovic, M, Arany, Z, Roew, GC, Souza, A, Cheng, S, McCade, EL, Yang, E, Shi, X, Deo, R, Roth, FP, Asnani, A, Rhee, EP, Systrom, DM, Semigran, MJ, Vasan, RS, Carr, SA, Wang, TJ, Sabatine, MS, Clish, CB & Gerszten, RE 2010, 'Metabolic signatures of exercise in human plasma', *Science Translational Medicine*, vol. 2, no. 33, pp. 33-37.

Lexell, J 1995, 'Human aging, muscle mass, and fiber type composition', *The Journals of Gerontology. Series A, Biological Sciences and Medical Sciences*, 1995, Spec No. 50, pp. 11-16.

Lira, FS, Carnevali, LC, Zanchi, NE, Santos, RVT, Lavoie, JM, & Seelaender, M 2012, 'Exercise intensity modulation of hepatic lipid metabolism', *Journal of Nutrition and Metabolism*, vol. 2012, e809576.

Lira, FS, Zanchi, NE, Lima-Silva, AE, Pires, FO, Bertuzzi, RC, Caperuto, EC, Kiss, MA, Seelaender, M, & Santos, RV 2010, 'Is acute supramaximal exercise capable of modulating lipoprotein profile in healthy men?', *European Journal of Clinical Investigation*, vol. 40, no. 8, pp. 759-765.

Lira, FS, Zanchi, NE, Lima-Silva, AE, Pires, FO, Bertuzzi, RC, Santos, RV, Caperuto, EC, Kiss, MA, & Seelaender, M 2009, 'Acute high-intensity exercise with low energy expenditure reduced LDL-c and total cholesterol in men', *European Journal of Applied Physiology*, vol. 107, no. 2, pp. 203-210.

Lister, R, Pelizzola, M, Dowen, RH, Hawkins, RD, Hon, G, Tonti-Filippini, J, Nery, JR, Lee, L, Ye, Z, Ngo, QM, Edsall, L, Antosiewicz-Bourget, J, Stewart, R, Millar, AH, Thomson, JA, Ren, B & Ecker, JR 2009, 'Human DNA methylomes at base resolution show widespread epigenomic differences', *Nature*, vol. 462, no. 7271, pp. 3150322.

Liu, G, Zhu, H, Lagou, V, Gutin, B, Stallmann-Jorgensen, IS, Treiber, FA, Dong, Y & Snieder, H 2010, 'FTO variant rs9939609 is associated with body mass index and waist circumference, but not with energy intake or physical activity in European- and African-American youth', *BMC Medical Genetics*, vol. 11, pp. 57.

Livak, KJ & Schmittgen, TD 2001, 'Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta CT}$ method', *Methods*, vol. 25, no. 4, pp. 402-408.

Locke, AE, Kahali, B, Berndt, SI, Justice, AE, Pers, TH et al. 2015, 'Genetic studies of body mass index yield new insights for obesity biology', *Nature*, vol. 518, no. 7538, pp. 197-206.

Lovejoy, J, Newby, FD, Gebhart, SS & DiGirolamo, M 1992, 'Insulin resistance in obesity is associated with elevated basal lactate levels and diminished lactate appearance following intravenous glucose and insulin', *Metabolism*, vol. 41, no. 1, pp. 22-27.

Ma, G, Wang, Y, Li, Y, Cui, L, Zhao, Y, Zhao, B & Li1, K 2015 'MiR-206, a key modulator of skeletal muscle development and disease', *International Journal of Biological Sciences*, vol. 11, no. 3, pp. 345–352.

Ma, M, Harding, HP, O'Rahilly, S, Ron, D & Yeo, GSH 2012, 'Kinetic analysis of FTO (fat mass and obesity-associated) reveals that it is unlikely to function as sensor for 2-oxoglutarate', *The Biochemical Journal*, vol. 444, no. 2, pp. 183-187.

Maes, HH, Neale, MC & Eaves, LJ 1997, 'Genetic and environmental factors in relative body weight and human obesity', *Behavior Genetics*, vol. 27, no. 4, pp. 325-351.

Mägi, R, Manning, S, Yousseif, A, Pucci, A, Santini, F, Karra, E, Querci, G, Pelosini, C, McCarthy, MI, Lindgren, CM & Batterham, RL 2013, 'Contribution of 32 GWAS-identified common variants to severe obesity in European adults referred for bariatric surgery', *PLoS One*, vol. 8, no. 8, e70735.

Malatesta, D, Werlen, C, Bulfaro, S, Chenevière, X, & Borrani, F 2009, 'Effect of high-intensity interval exercise on lipid oxidation during postexercise recovery', *Medicine and Science in Sports and Exercise*, vol. 41, no. 2, pp. 364-374.

Matsuda, M & DeFronzo, RA 1999, 'Insulin sensitivity indices obtained from oral glucose tolerance testing: comparison with the euglycemic insulin clamp', *Diabetes Care*, vol. 22, no. 9, pp. 1462-1470.

Matthews, DR, Hosker, JP, Rudenski, AS, Naylor, BA, Treacher, DF & Turner RC 1985, 'Homeostasis model assessment: insulin resistance and beta-cell function from fasting plasma glucose and insulin concentrations in man', *Diabetologia*, vol. 28, no. 7, pp: 412-419.

Maughan, RJ, Whiting, PH, & Davidson, RJ 1985, 'Estimation of plasma volume changes during marathon running', *British Journal of Sports Medicine*, vol. 19, no. 3, pp. 138-141.

Mayer, J, Roy, P & Mitra, KP 1956, 'Relation between caloric intake, body weight, and physical work: studies in an industrial male population in West Bengal', *American Journal of Clinical Nutrition*, vol. 4, no. 2, pp. 169-175.

McCaffery, JM, Papandonatos, GD, Peter, I, Huggins, GS, Raynor, HA, Delahanty, LM, Cheskin, LJ, Balasubramanyam, A & Wagenknecht, LE 2012, 'Obesity susceptibility loci and dietary intake in the look ahead trial', *The American Journal of Clinical Nutrition*, vol. 95, no. 6, pp. 1477-1486.

McCarthy, JJ & Esser, KA 2007, 'MicroRNA-1 and microRNA-133a expression are decreased during skeletal muscle hypertrophy', *Journal of Applied Physiology*, vol. 102, no. 1, pp. 306-313.

McCoy, M, Proietto, J & Hargreaves, M 1996, 'Skeletal muscle GLUT-4 and postexercise muscle glycogen storage in humans', *Journal of Applied Physiology*, vol. 80, no. 2, pp. 411-415.

McMurray, F, Church, CD, Larder, R, Nicholson, G, Wells, S, Teboul, L, Tung, YCL, Rimmington, D, Bosch, F, Jimenez, V, Yeo, GSH, O'Rahilly, S, Ashcroft, FM, Coll, AP & Cox, RD 2013, 'Adult onset global loss of the Fto gene alters body composition and metabolism in the mouse', *PLoS Genetics*, vol. 9, no. 1, e1003166.

McTaggart, JS, Lee, S, Iberi, M, Church, C, Cox, RD & Ashcroft, FM 2011, 'FTO is expressed in neurones throughout the brain and its expression is unaltered by fasting', *PLoS One*, vol. 6, no. 11, e27968.

Merkestein, M, McTaggart, JS, Lee, S, Kramer, HB, McMurray, F, Lafond, M, Boutens, L, Cox, R & Ashcroft, FM 2014, 'Changes in gene expression associated with FTO overexpression in mice', *PLoS One*, vol. 9, no. 5, e97162.

Meyer, KD, Saletore, Y, Zumbo, P, Elemento, O, Mason, CE & Jaffrey, SR 2012, 'Comprehensive analysis of mRNA methylation reveals enrichment in 3′ UTRs and near stop codons', *Cell*, vol. 149, no. 7, pp. 1635-1646.

Michael, LF, Wu, Z, Cheatham, RB, Puigserver, P, Adelmant, G, Lehman, JJ, Kelly, DP & Spiegelman, BM 2001, 'Restoration of insulin-sensitive glucose transporter (GLUT4) gene expression in muscle cells by the transcriptional coactivator PGC-1', *Proceedings of the National Academy of Sciences of the United States of America*, vol. 98, no. 7, pp. 3820-3825.

Milagro, FI, Campión, J, Cordero, P, Goyenechea, E, Gómez-Uriz, AM, Abete, I, Zulet, MA & Martínez, JA 2011, 'A dual epigenomic approach for the search of obesity biomarkers: DNA methylation in relation to diet-induced weight loss', *FASEB Journal: Federation of American Societies for Experimental Biology*, vol. 25, no. 4, pp. 1378-1389.

Mittendorfer, B 2011, 'Origins of metabolic complications in obesity: adipose tissue and free fatty acid trafficking', *Current Opinion in Clinical Nutrition and Metabolic Care*, vol. 14, no. 6, pp. 535-541.

Morgan, DK & Whitelaw, E 2008, 'The case for transgenerational epigenetic inheritance in humans', *Mammalian Genome*, vol. 19, no. 6, pp. 394-397.

Morton, GJ, Cummings, DE, Baskin, DG, Barsh, GS & Schwartz, MW 2006, 'Central nervous system control of food intake and body weight', *Nature*, vol. 443, no. 7109, pp. 289-295.

Mourtzakis, M, Saltin, B, Graham, T, & Pilegaard, H 2006, 'Carbohydrate metabolism during prolonged exercise and recovery: interactions between pyruvate dehydrogenase, fatty acids, and amino acids', *The Journal of Applied Physiology*, vol. 100, no. 6, pp. 1822-1830.

Mulla, NA, Simonsen, L, & Bülow, J 2000, 'Post-exercise adipose tissue and skeletal muscle lipid metabolism in humans: the effects of exercise intensity', *The Journal of Physiology*, vol. 524, no. 3, pp. 919-928.

Müller, TD, Tschöp, MH & Hofmann, S 2013, 'Emerging function of fat mass and obesity-associated protein (Fto)', *PLoS Genetics*, vol. 9, no. 1, e1003223.

Mustelin, L, Silventoinen, K, Pietiläinen, K, Rissanen, A & Kaprio, J 2009, 'Physical activity reduces the influence of genetic effects on BMI and waist circumference: a study in young adult twins', *International Journal of Obesity (London)*, vol. 33, no. 1, pp. 29-36.

Nicholson, JK, Lindon, JC, & Holmes, E 1999, 'Metabonomic: understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data', *Xenobiotica*, vol. 29, no. 11, pp. 1181-1189.

Nitert, MD, Dayeh, T, Volkov, P, Elgzyri, T, Hall, E, Nilsson, E, Yang, BT, Lang, S, Parikh, H, Wessman, Y, Weishaupt, H, Attema, J, Wierup, N, Almgren, P, Jansson, PA, Rönn, T, Hansson, O, Eriksson, KF, Groop, L & Ling, C 2012, 'Impact of an exercise intervention on DNA methylation in skeletal muscle from first-degree relatives of patients with type 2 diabetes', *Diabetes*, vol. 61, no. 12, pp. 3322-3332.

Niu, Y, Zhao, X, Wu, YS, Li, MM, Wang, XJ & Yang, YG 2013, 'N^6-methyl-adenosine (m^6A) in RNA: An old modification with a novel epigenetic function', *Genomics Proteomics Bioinformations*, vol. 11, no. 1, pp. 8-17.

Ollikainen, M, Smith, KR, Joo, EJ, Ng, HK, Andronikos, R, Novakovic, B, Abdul Aziz, NK, Carlin, JB, Morley, R, Saffery, R, & Craig, JM 2010, 'DNA methylation analysis of multiple tissues from newborn twins reveals both genetic and intrauterine components to variation in the human neonatal epigenome', *Human Molecular Genetics*, vol. 19, no. 21, pp. 4176-4188.

Palla, L, Higgins, JP, Wareham, NJ & Sharp, SJ 2010, 'Challenges in the use of literature-based meta-analysis to examine gene-environment interactions', *American Journal of Epidemiology*, vol. 171, no. 11, pp. 1225-1232.

Pan, T 2013, 'N^6-methyl-Adenosine modification in messenger and long non-coding RNA', *Trends in Biomedical Science*, vol. 38, no. 4, pp. 204-209.

Park, S, Sadanala, KC & Kim, EK 2015, 'A metabolomic approach to understanding the metabolic link between obesity and diabetes', *Molecules and Cells*, vol. 38, no. 7, pp. 587-596.

Peake, JM, Tan, SJ, Markworth, JF, Broadbent, JA, Skinner, TL, & Cameron-Smith, D 2014, 'Metabolic and hormonal responses to isoenergetic high-intensity interval exercise and continuous moderate-intensity exercise', *American Journal of Physiology, Endocrinology and Metabolism*, vol. 307, no.7, E339-352.

Perry, CG, Lally, J, Holloway, GP, Heigenhauser, GJ, Bonen, A & Spriet, LL 2010, 'Repeated transient mRNA bursts precede increases in transcriptional and mitochondrial proteins during training in human skeletal muscle', *The Journal of Physiology*, vol. 1, no. 588 (Pt 23), pp. 4795-4810.

Peter, T, Ausmeier, K & Rüther, U 1999, 'Cloning of fatso (Fto), a novel gene deleted by the fused toes (Ft) mouse mutation', *Mammalian Genome*, vol. 10, no. 10, pp. 983-986.

Phillips, CM, Kesse-Guyot, E, McManus, R, Hercberg, S, Lairon, D, Planells, R & Roche, HM 2012, 'High dietary saturated fat intake accentuates obesity risk associated with the fat mass and obesity-associated gene in adults', *The Journal of Nutrition*, vol. 142, no. 5, pp. 824-31.

Poritsanos, NJ, Lew, PS, Fischer, J, Mobbs, CV, Nagy, JI, Wong, D, Rüther, U & Mizuno, TM 2011, 'Impaired hypothalamic Fto expression in response to fasting and glucose in obese mice', *Nutrition and Diabetes*, vol. 1: e19.

Poritsanos, NJ, Lews, PS & Mizuno, TM 2010, 'Relationship between blood glucose levels and hepatic Fto mRNA expression in mice', *Biochemical and Biophysical Research Communications*, vol. 400, no. 4, pp. 713-713.

Portela, A & Esteller, M 2010, 'Epigenetic modifications and human disease', *Nature Biotechnology*, vol. 28, no. 10, pp. 1057-1068.

Prior, SJ, Ryan, AS, Stevenson, TG & Goldberg, AP 2014, 'Metabolic inflexibility during submaximal aerobic exercise is associated with glucose intolerance in obese older adults', *Obesity (Silver Spring)*, vol. 22, no. 2, pp. 451-457.

Qi, L, Kang, K, Zhang, C, van Dam, RM, Kraft, P, Hunter, D, Lee, CH & Hu, FB 2008, 'Fat mass-and obesity-associated (FTO) gene variant is associated with obesity: longitudinal analyses in two cohort studies and functional test', *Diabetes*, vol. 57, no. 11, pp. n3145-3151.

Qi, Q, Downer, MK, Kilpeläinen, TO, Taal, HR, Barton, SJ, Ntalla, I, Standl, M, Boraska, V, Huikari, V, Kiefte-de Jong, JC, Körner, A, Lakka, TA, Liu, G, Magnusson, J, Okuda, M, Raitakari, O, Richmond, R, Scott, RA, Bailey, ME, Scheuermann, K, Holloway, JW, Inskip, H, Isasi, CR, Mossavar-Rahmani, Y, Jaddoe, VW, Laitinen, J, Lindi, V, Melén, E, Pitsiladis, Y, Pitkänen, N, Snieder, H, Heinrich, J, Timpson, NJ, Wang, T, Yuji, H, Zeggini, E, Dedoussis, GV, Kaplan, RC, Wylie-Rosett, J, Loos, RJ, Hu, FB & Qi, L 2015, 'Dietary intake, FTO genetic variants, and adiposity: A combined analysis of over 16,000 children and adolescents', *Diabetes*, vol. 64, no. 7, pp. 2467-2476.

Rampersaud, E, Mitchell, BD, Pollin, TI, Shen, H, O'Connell, JR, Ducharme, JL, Hines, S, Sack, P, Naglieri, R, Shuldiner, AR & Snitker, S 2008, 'Physical activity and the association of common FTO gene variants with body mass index and obesity', *Archives of Internal Medicine*, vol. 168, no. 16, pp. 1791-1717.

Ravussin, E, Valencia, ME, Esparza, J, Bennett, PH & Schulz, LO 1994, 'Effects of a traditional lifestyle on obesity in Pima Indians', *Diabetes Care*, vol. 17, no. 9, pp. 1067-1074.

Rokholm, B, Silventoinen, K, Tynelius, P, Gamborg, M, Sørensen, TIA & Rasmussen, F 2011, 'Increasing genetic variance of body mass index during the Swedish obesity epidemic', *PLoS One*, vol. 6, no. 11, e27135.

Rolland-Cachera, MF, Deheeger, M, Maillot, M & Bellisle, F 2006, 'Early adiposity rebound: causes and consequences for obesity in children and adults', *International Journal of Obesity (London)*, vol. 30, no. 4, pp. S11-17.

Romijn, J, Coyle, E, Sidossis, L, Gastaldelli, A, Horowitz, J, Endert, E, & Wolfe. R 1993, 'Regulation of endogenous fat and carbohydrate metabolism in relation to exercise intensity and duration', *American Journal of Physiology, Endocrinology and Metabolism*, vol. 265, no.1, E380-391.

Rönn, T, Volkov, P, Davegårdh, C, Dayeh, T, Hall, E, Olsson, AH, Nilsson, E, Tornberg, Å, Dekker Nitert, M, Eriksson, KF, Jones, HA, Groop, L & Ling, C 2013, 'A six months exercise intervention influences the genome-wide DNA methylation pattern in human adipose tissue', *PLoS Genetics*, vol. 9, no. 6, e1003572.

Rose, AJ & Richter, EA 2005, 'Skeletal muscle glucose uptake during exercise: how is it regulated?', *Physiology (Bethesda)*, vol. 20, pp. 260-270.

Saldaña-Alvarez, Y, Salas-Martínez, MG, García-Ortiz, H, Luckie-Duque, A, García-Cárdenas, G, Vicenteño-Ayala, H, Cordova, EJ, Esparza-Aguilar, M, Contreras-Cubas, C, Carnevale, A, Chávez-Saldaña, M & Orozco, L 2016, 'Gender-dependent association of FTO polymorphisms with body mass index in Mexicans', *PLoS One*, vol. 11, no. 1, e0145984.

Selak, MA, Armour, SM, MacKenzie, ED, Boulahbel, H, Watson, DG, Mansfield, KD, Pan, Y, Simon, MC, Thompson, CB & Gottileb, E 2005, 'Succinate links TCA cycle dysfunction to oncogenesis by inhibiting HIF-alpha prolyl hydroxylase', *Cancer Cell*, vol. 7, no. 1, pp. 77-85.

Senekal, M 2012, 'Genotype-based personalised nutrition for obesity prevention and treatment: are we there yet?', *The Scientific World Journal*, vol. 25, no. 1, pp. 9-14.

Solomon, TPJ, Marchetti, CM, Krishnan, RK, Gonzalez, F & Kirwan, JP 2008, 'Effects of aging on basal fat oxidation in obese humans', *Metabolism*, vol. 57, no. 8, pp. 1141–1147.

Sonestedt, E, Roos, C, Gullberg, B, Ericson, U, Wirfält, E & Orho-Melander, M 2009, 'Fat and carbohydrate intake modify the association between genetic variation in the FTO genotype and obesity', *The American Journal of Clinical Nutrition*, vol. 90, no. 5, pp. 1418-1425.

Sovio, U, Mook-Kanamori, DO, Warrington, NM, Lawrence, R, Briollais, L, Palmer, CAN, Cecil, J, Sandling, JK, Syvänen, AC, Kaakinen, M, Beilin, LJ, Millwood, IY, Bennet, AJ, Laitinen, J, Pouta, A, Molitor, J, Davey Smith, G, Ben-Shlomo, Y, Jaddoe, VW, Palmer, LJ, Pennell, CE, Cole, TJ, McCarthy, MI, Järvelin, MR, Timpson, NJ & Early Growth Genetics Consortium 2011, 'Association between common variation at the FTO locus and changes in body mass index from infancy to late childhood: the complex nature of genetic association through growth and development', *PLoS Genetics*, vol. 7, no. 2, e1001307.

Speakman, JR, Rance, KA & Johnstone, AM 2008, 'Polymorphisms of the FTO gene are associated with variation in energy intake, but not energy expenditure', *Obesity (Silver Spring)*, vol. 16, no. 8, pp. 1961-1965.

Spector, DL & Lamond, AI 2011, 'Nuclear Speckles', *Cold Spring Harbour Perspectives Biology*, vol. 3, no. 2, a000646.

Speliotes, EK, Willer, CJ, Berndt, SI, Monda, KL, Thorleifsson, G, et al. 2010, 'Association analyses of 249,796 individuals reveal 18 new loci associated with body mass index', *Nature Genetics*, vol. 42, no. 11, pp. 937-948.

Storlien, L, Oakes, ND & Kelley, DE 2004, 'Metabolic flexibility', *The Proceedings of the Nutrition Society*, vol. 63, no. 2, pp. 363-368.

Stratigopoulos, G, Padilla, SL, LeDuc, CA, Watson, E, Hattersley, AT, McCarthy, MI, Zeltser, LM, Chung, WK & Leibel, RL 2008, 'Regulation of Fto/Ftm gene expression in mice and humans', *American Journal of Physiology: Regulatory Integrative and Comparative Physiology*, vol. 294, no. 4, pp. 1185-1196.

Stuart, CA, Howell, MEA, Baker, JD, Dykes, RJ, Duffourc, MM, Ramsey, MW & Stone, MH 2010, 'Cycle training increased GLUT4 and activation of mTOR in fast twitch muscle fibers', *Medicine and Science in Sports and Exercise*, vol. 42, no. 1, pp. 96–106.

Stubbs, RJ 1999, 'Peripheral signals affecting food intake', *Nutrition*, vol. 15, no. 7-8, pp. 614-625.

Tanofsky-Kraff, M, Han, JC, Anandalington, K, Shomaker, LB, Columbo, KM, Wolkoff, LE, Kozlosky, M, Elliot, C, Ranzenhofer, LM, Roza, CA, Yanovski, SZ & Yanovski, JA 2009, 'The FTO gene rs9939609 obesity-risk allele and loss of control over eating', *The American Journal of Clinical Nutrition*, vol. 90, no. 6, pp. 1483-1488.

Thomas, PR 1991, 'Improving America's diet and health: from recommendations to action', Committee of Dietary Guidelines Implementation: Institute of Medicine, National Academy Press, Washington D.C.

Timpson, NJ, Emmett, PM, Frayling, TM, Rogers, I, Hattersley, AT, McCarthy, MI & Davey Smith, G 2008, 'The fat mass- and obesity-associated locus and dietary intake in children', *The American Journal of Clinical Nutrition*, vol. 88, no. 4, pp. 971-978.

Trapp, E, Chisolm, D, Freund, J, & Boutcher, S 2008, 'The effects of high-intensity intermittent exercise training on fat loss and fasting insulin levels of young women', *International Journal of Obesity*, vol. 32, no. 4, pp. 684-691.

Tremblay, A, Despres, JP, Leblanc, C, Craig, CL, Ferris, B, Stephens, T, & Bouchard, C 1990, 'Effect of intensity of physical activity on body fatness and fat distribution', *American Journal of Clinical Nutrition*, vol. 51, no. 2, pp. 153-157.

Tschöp, MH, Speakman, JR, Arch, JR, Auwerx, J, Brüning, JC, Chan, L, Eckel, RH, Farese, RV Jr, Galgani, JE, Hambly, C, Herman, MA, Horvath, TL, Kahn, BB, Kozma, SC, Maratos-Flier, E, Müller, TD, Münzberg, H, Pfluger, PT, Plum, L, Reitman, ML, Rahmouni, K, Shulman, GI, Thomas, G, Kahn, CR & Ravussin, E 2011, 'A guide to analysis of mouse energy metabolism', *Nature Methods*, vol. 9, no. 1, pp. 57-63.

Tsekouras, YE, Magkos, F, Kellas, Y, Basioukas, KN, Kavouras, SA, & Sidossis, LS 2008, 'High-intensity interval aerobic training reduces hepatic very low-density lipoprotein-triglyceride secretion rate in men', *American Journal of Physiology, Endocrinology and Metabolism*, vol. 295, no. 4, E851-858.

van de Weijer, T, Sparks, LM, Phielix, E, Meex, RC, van Herpen, NA, Hesselink, MK, Schrauwen, P & Schrauwen-Hinderling, VB 2013, 'Relationships between mitochondrial function and metabolic flexibility in type 2 diabetes mellitus', *PLoS One*, vol. 8, no. 2, e51648.

van Loon, LJC, Greenhaff, PL, Constantin-Teodosiu, D, Saris, WH, & Wagenmakers, JM 2001, 'The effects of increasing exercise intensity on muscle fuel utilisation in humans', *The Journal of Physiology*, vol. 536, no. 1, pp. 295-304.

van Ommen, B, Keiger, J, Heil, SG & Kaput, J 2009, 'Challenging homeostasis to define biomarkers for nutrition related health', *Molecular Nutrition and Food Research*, vol. 53, no. 7, pp. 795-804.

Vierkotten, J, Dildrop, R, Peters, T, Wang, B & Rüther, U 2007, 'Ftm is a novel basal body protein of cilia involved in Shh signalling', *Development*, vol. 134, no. 14, pp. 2569-2577.

Vimaleswaran, KS, Li, S, Zhao, JH, Luan, J, Bingham, SA, Khaw, KT, Ekelund, U, Wareham, NJ & Loos, RJ 2009, 'Physical activity attenuates the body mass index-increasing influence of genetic variation in the FTO gene', *The American Journal of Clinical Nutrition*, vol. 90, no. 2, pp. 425-428.

Wahl, S, Krug, S, Then, C, Kirchhofer, A, Kastenmüller, G, Brand, T, Skurk, T, Claussnitzer, M, Huth, C, Heier, M, Meisinger, C, Peters, A, Thorand, B, Gieger, C, Prehn, C, Römisch-Margi, W, Adamski, J, Suhre, K, Illig, T, Grallert, H, Lauman, H, Seissler, J & Hauner, H 2014, 'Comparative analysis of plasma metabolomics response to metabolic challenge tests in healthy subjects and influence of the FTO obesity risk allele', *Metabolomics*, vol. 10, pp. 386-401.

Wang, P, Yang, FJ, Du, H, Guan, YF, Xu, TY, Xu, XW, Su, DF & Miao, CY 2011, 'Involvement of leptin receptor long isoform (LepRb)-STAT3 signaling pathway in brain fat mass- and obesity-associated (FTO) downregulation during energy restriction', *Molecular Medicine*, vol. 17, no. 5-6, pp. 523-532.

Wang, X, Lu, Z, Gomez, A, Hon, GC, Yue, Y, Han, D, Fu, Y, Parisien, M, Dai, Q, Jia, G, Ren, B, Pan, T & He, C 2014, 'm^6A-dependent regulation of messenger RNA stability', *Nature*, vol. 505, no. 7481, 10.1038/nature12730.

Wang, X, Zhu, H, Snieder, H, Su, S, Munn, D, Harshfield, G, Maria, BL, Dong, Y, Treiber, F, Gutin, B & Shi, H 2010, 'Obesity related methylation changes in DNA of peripheral blood leukocytes', *BMC Medicine*, vol. 8, pp. 88.

Wardle, J, Carnell, S, Haworth, CM, Farooqi, IS, O'Rahilly, S, & Plomin, R 2008, 'Obesity associated genetic variation in FTO is associated with diminished satiety', *The Journal of Clinical Endocrinology and Metabolism*, vol. 93, no. 9, pp. 3640-3643.

Wardle, J, Llewellyn, C, Sanderson, S & Plomin, R 2009, 'The FTO gene and measured food intake in children', *International Journal of Obesity (London)*, vol. 33, no. 1, pp. 42-45.

Waterland, RA & Jirtle, RL 2003, 'Transposable elements: targets for early nutritional effects on epigenetic gene regulation', *Molecular and Cellular Biology*, vol. 23, no. 15, pp. 5293-5300.

Westerhuis, JA, van Velzen, EJ, Hoefsloot, HC, & Smilde, AK 2010, 'Multivariate paired data analysis: multilevel PLSDA versus OPLSDA', *Metabolomics*, vol. 6, no. 1, pp.119-128.

Willer, CJ, Sanna, S, Jackson, AU, Scuteri, A, Bonnycastle, LL, Clarke, R, Heath, SC, Timpson, NJ, Najjar, SS, Stringham, HM, Strait, J, Duren, WL, Maschio, A, Busonero, F, Mulas, A, Albai, G, Swift, AJ, Morken, MA, Narisu, N, Bennett, D, Parish, S, Shen, H, Galan, P, Meneton, P, Hercberg, S, Zelenika, D, Chen, WM, Li, Y, Scott, LJ, Scheet, PA, Sundvall, J, Watanabe, RM, Nagaraja, R, Ebrahim, S, Lawlor, DA, Ben-Shlomo, Y, Davey-Smith, G, Shuldiner, AR, Collins, R, Bergman, RN, Uda, M, Tuomilehto, J, Cao, A, Collins, FS, Lakatta, E, Lathrop, GM, Boehnke, M, Schlessinger, D, Mohlke, KL & Abecasis, GR 2008, 'Newly identified loci that influence lipid concentrations and risk of coronary artery disease', *Nature Genetics*, vol. 40, no. 2, pp. 161-169.

Willoughby, DS, Stout, JR & Wilborn, CD 2007, 'Effects of resistance training and protein plus amino acid supplementation on muscle anabolism, mass, and strength', *Amino Acids*, vol. 32, no. 4, pp. 467-477.

Wishart, DS, Jewison, T, Guo, AC, Wilson, M, Knox, C, Liu, Y, Djoumbou, Y, Mandal, R, Aziat, F, Dong, E, Bouatra, S, Sinelnikov, I, Arndt, D, Xia, J, Liu, P, Yallou, F, Bjorndahl, T, Perez-Pineiro, R, Eisner, R, Allen, F, Neveu, V, Greiner, R & Scalbert, A 2013, 'HMDB 3.0-The human metabolome database in 2012', *Nucleic Acids Research*, vol. 28, pp. 27-30.

Wold S, Ruhe, A, Wold, H & Dunn, WJJ 1984, 'The collinearity problem in linear regression. The partial least squares (PLS) approach to generalized inverses', *SIAM Journal of Scientific and Statistical Computing*, vol. 5, no. 3, pp. 735-743.

World Health Organisation (WHO) 2000, 'Obesity: preventing and managing the global epidemic', *WHO Technical Report Series*, vol. 894, pp. 5-13.

World Medical Organization (WMO) 1996, 'Declaration of Helsinki 1996', *British Medical Journal*, vol. 313, no. 7070, pp. 1448-1449.

Worley, B & Powers, R 2013, 'Multivariate analysis in metabolomics', *Current Metabolomics*, vol. 1, no. 1, pp. 92–107.

Wu, Q, Saunders, RA, Szkudlarek-Mikho, M, Serna Ide, L & Chin, KV 2010, 'The obesity-associated FTO gene is a transcriptional co-activator', *Biochemical and Biophysical Research Communications*, vol. 401, no. 3, pp. 390-395.

Xi, B, Gu, H, Baniasadi, H & Raftery, D 2014, 'Statistical analysis and modeling of mass spectrometry-based metabolomics data', *Methods in Molecular Biology*, vol. 1198, pp. 333–353.

Xi, B, Zhang, M, Wang, C, Shen, Y, Zhao, X, Wang, X, & Mi, J 2013, 'The common SNP (rs9939609) in the FTO gene modifies the association between obesity and high blood pressure in Chinese children', *Molecular Biology Reports*, vol. 40, no. 2, pp. 773-778.

Xia, J, Mandal, R, Sinelnikov, I, Broadhurst, D, & Wishart, DS 2012, 'MetaboAnalyst 2.0 - a comprehensive server for metabolomic data analysis', *Nucleic Acids Research*, vol. 40, W127-W133.

Xia, J, Sinelnikov, IV, Han, B & Wishart, DS 2015, 'MetaboAnalyst 3.0 – making metabolomics more meaningful', *Nucleic Acids Research*, vol. 43, W251-W257.

Xiang, L, Wu, H, Pan, A, Patel, B, Xiang, G, Qi, L, Kaplan, RC, Hu, F, Wylie-Rosett, J & Qi, Q 2016, 'FTO genotype and weight loss in diet and lifestyle interventions: a systematic review and meta-analysis', *American Journal of Clinical Nutrition*, vol. 103, no. 4, pp. 1162-1170.

Xiao, M, Yang, H, Xu, W, Ma, S, Lin, H, Zhu, H, Liu, L, Liu, Y, Yang, C, Xu, Y, Zhao, S, Ye, D, Xiong, Y & Guan, KL 2012, 'Inhibition of α-KG-dependent histone and DNA demethylases by fumarate and succinate that are accumulated in mutations of FH and SDH tumor suppressors', *Genes & Development*, vol. 26, no. 12, pp. 1326-1338.

Yako, YY, Echouffo-Tcheugui, JB, Balti, EV, Matsha, TE, Sobngwi, E, Erasmus, RT, Kengne, AP 2015, 'Genetic association studies of obesity in Africa: a systematic review', *Obesity Reviews*, vol. 16, no. 3, pp. 259-272.

Yang, J, Loos, RJ, Powell, JE, Medland, SE, Speliotes, EK, Chasman, DI, Rose, LM, Thorleifsson, G, Steinthorsdottir, V, Mägi R, Waite, L, Smith, AV, et al. 2012, 'FTO genotype is associated with phenotypic variability of body mass index', *Nature*, vol. 490, no. 7419, pp. 267–272.

Yoshioka, M, Doucet, E, St-Pierre, S, Alméras, N, Richard, D, Labrie, A, Després, JP, Bouchard, C, & Tremblay, A 2001, 'Impact of high-intensity exercise on energy expenditure, lipid oxidation and body fatness', *International Journal of Obesity Related and Metabolic Disorders*, vol. 25, no. 3, pp. 332-339.

Zabena, C, González-Sánchez, JL, Martinez-Larrad, MT, Torres-Garcia, A, Alvarez-Fernández-Represa, J, Corbatón-Anchuelo, A, Pérez-Barba, M & Serrano-Rios, M 2009, 'The FTO obesity gene. Genotyping and gene expression analysis in morbidly obese patients', *Obesity Surgery*, vol. 19, no. 1, pp. 87-95.

Zhang, M, Zhang, Y, Ma, J, Guo, F, Cao, Q, Zhang, Y, Zhou, B, Chai, J, Zhao, W & Zhao, R 2015, 'The demethylase activity of FTO (Fat Mass and Obesity Associated Protein) is required for preadipocyte differentiation', *PLoS One*, vol. 10, no. 7, e0133788.

Zhao, X, Ying, Y, Sun, BF, Shi, Y, Yang, X, Xiao, W, Hao, YJ, Ping, XL, Chen, YS, Wang, W-J, Jin, KX, Wang, X, Huang, CM, Fu, Y, Ge, XM, Song, SH, Jeong, HS, Yanagisawa, H, Niu, Y, Jia, GF, Wu, W, Tong, WM, Okamoto, A, He, C, Danielsen, JMR, Wang, XJ & Yang, YG 2014, 'FTO-dependent demethylation of N^6-methyadenosine regulates mRNA splicing and is required for adipogenesis', *Cell*, vol. 24, no. 12, pp. 1403-1419.

Zheng, G, Dahl, JA, Niu, Y, Fedorcsak, P, Huang, CM, Li, CJ, Vågbø, CB, Shi, Y, Wang, WL, Song, SH, Lu, Z, Bosmans, RP, Dai, Q, Hao, YJ, Yang, X, Zhao, WM, Tong, WM, Wang XJ, Bogdan, F, Furu, K, Fu, Y, Jia, G, Zhao, X, Liu, J, Krokan, HE, Klungland, A, Yang, YG & He, C 2013, 'ALKBH5 is a mammalian RNA demethylase that impacts RNA metabolism and mouse fertility', *Molecular Cell*, vol. 49, no. 1, pp. 18-29.

Zhou, J, Wan, J, Gao, X, Zhang, X, Jaffrey, SR & Qian, SB 2015, 'Dynamic m^6A mRNA methylation directs translational control of heat shock response', *Nature*, vol. 536, no. 7574, pp. 591-594.

Zierath, JR & Hawley, JA 2004 'Skeletal muscle fiber type: influence on contractile and metabolic properties', *PLoS Biology*, vol. 2, no. 10, e348.

Zurlo, F, Lillioja, S, Esposito-Del, Puente, A, Nyomba, BL, Raz, I, Saad, MF, Swinburn, BA, Knowler, WC, Bogardus, C & Ravussin, E 1990, 'Low ratio of fat to carbohydrate oxidation as predictor of weight gain: study of 24-h RQ', *The American Journal of Physiology*, vol. 259, no. 5 Pt 1, E650-657.

Diet and Activity/ Exercise Log

Name:___________________________________ **Date/Week:**_______________

Instructions:

1. Record everything you eat for 4 days (including 1 weekend day).
2. Record amounts in grams, cups, mls or individual amounts as best as possible, rather than referring to 'one bowl' or 'half a glass'. Also refer to the method of preparation. Examples include: 200g baked vs. fried chicken, 1 cup of rice, 2 tsp of butter, 1.5 cups of 2% milk.
3. Record immediately after eating. Waiting until night may make it hard to remember specific details.

Time	Food/Beverage	Amount	Kilojoules (optional)	Fat (grams) (optional)

Activity/Exercise Type	Duration

Figure S-3.1 Diet and Activity/Exercise Log. Blank template of record completed over 4 days prior to participants undertaking experimental trial protocols detailed in Chapter 4.

Gender	Male	Female	*P value*
n	50	97	
Age (yr)	28.1 ± 1.0	29.5 ± 0.9	0.314
Total Body Mass (kg)	78.2 ± 2.0	66.1 ± 1.1	**<0.001**
Height (cm)	177.5 ± 1.1	164.7 ± 0.7	**<0.001**
BMI (kg/m^2)	24.7 ± 0.5	24.4 ± 0.4	0.649
Hip Circumference (cm)	100.6 ± 1.2	101.4 ± 0.9	0.567
Waist Circumference (cm)	82.5 ± 1.4	74.6 ± 0.9	**<0.001**
Fat Mass (%)	18.2 ± 0.9	31.6 ± 0.7	**<0.001**
Muscle Mass (kg)	60.2 ± 1.2	42.4 ± 0.4	**<0.001**
Water (%)	58.1 ± 0.7	50.6 ± 0.5	**<0.001**
Systolic BP (mmHg)	130.1 ± 1.7	118.8 ± 1.3	**<0.001**
Diastolic BP (mmHg)	75.5 ± 1.2	73.4 ± 0.9	0.180

Table S-4.1 Clinical characteristics of the studied cohort based on their gender. Values are expressed as mean ± SEM. Total body mass, height, waist circumference, muscle mass, water mass and systolic BP were significantly higher in males compared to females (bolded, $p < 0.05$). Males had significantly lower fat mass than females ($p < 0.001$).

	Genotype			
Males (n=50)	**AA**	**AT**	**TT**	***P value***
n	9	24	17	
Age (yr)	30.0 ± 3.0	26.9 ± 1.0	28.7 ± 2.0	0.475
Total Body Mass (kg)	78.9 ± 3.8	76.5 ± 2.1	80.2 ± 4.7	0.703
Height (cm)	178.7 ± 2.8	178.2 ± 1.5	175.8 ± 1.9	0.539
BMI (kg/m^2)	24.7 ± 1.1	24.0 ± 0.5	25.7 ± 1.1	0.365
Hip Circumference (cm)	101.6 ± 2.3	99.9 ± 1.2	102.0 ± 2.5	0.651
Waist Circumference (cm)	82.4 ± 2.6	80.4 ± 1.5	84.4 ± 3.0	0.409
Fat Mass (%)	18.8 ± 1.3	16.8 ± 1.1	20.0 ± 1.8	0.253
Muscle Mass (kg)	60.8 ± 2.8	60.1 ± 1.2	60.1 ± 2.7	0.973
Water (%)	57.4 ± 1.0	59.0 ± 0.9	57.0 ± 1.5	0.401
Systolic BP (mmHg)	134.3 ± 4.1	129.7 ± 2.1	128.4 ± 3.4	0.484
Diastolic BP (mmHg)	79.0 ± 2.8	73.5 ± 1.7	76.5 ± 2.2	0.225
Females (n=97)	**AA**	**AT**	**TT**	
n	23	38	36	
Age (yr)	29.7 ± 2.1	29.9 ± 1.5	29.0 ± 1.4	0.908
Total Body Mass (kg)	67.3 ± 2.6	66.3 ± 1.7	65.0 ± 1.7	0.716
Height (cm)	165.3 ± 1.7	164.4 ± 1.0	164.7 ± 1.3	0.887
BMI (kg/m^2)	24.6 ± 0.8	24.7 ± 0.7	24.0 ± 0.6	0.735
Hip Circumference (cm)	102.3 ± 2.1	101.9 ± 1.3	100.4 ± 1.4	0.643
Waist Circumference (cm)	75.8 ± 2.1	74.9 ± 1.5	73.6 ± 1.1	0.624
Fat Mass (%)	31.0 ± 1.7	32.4 ± 1.1	30.9 ± 1.1	0.607
Muscle Mass (kg)	43.2 ± 1.1	42.1 ± 0.6	42.2 ± 0.7	0.611
Water (%)	50.8 ± 1.2	50.1 ± 0.8	51.2 ± 0.8	0.634
Systolic BP (mmHg)	117.0 ± 2.0	118.5 ± 2.0	120.2 ± 2.6	0.657
Diastolic BP (mmHg)	74.8 ± 2.0	72.2 ± 1.4	73.9 ± 1.4	0.497

Table S-4.2 Clinical characteristics of the studied cohort based on their FTO rs9939609 genotype and separated for gender. Values are expressed as mean ± SEM.

Outcome (between FTO genotypes)	R^2	*P* value
Covariant: BMI		
Metabolic Flexibility	0.020	0.273
Energy Expenditure (kJ.kgLBM^{-1}.min^{-1}) – Pre OGL	0.004	0.908
Energy Expenditure (kJ.kgLBM^{-1}.min^{-1}) – Post OGL	0.011	0.474
Carbohydrate Oxidation (g.kgLBM^{-1}.min^{-1}) – Pre OGL	0.061	0.882
Carbohydrate Oxidation (g.kgLBM^{-1}.min^{-1}) – Post OGL	0.002	0.922
Fat Oxidation (g.kgLBM^{-1}.min^{-1}) – Pre OGL	0.072	0.541
Fat Oxidation (g.kgLBM^{-1}.min^{-1}) – Post OGL	0.009	0.619
Covariant: Gender		
Metabolic Flexibility	0.019	0.267
Energy Expenditure (kJ.kgLBM^{-1}.min^{-1}) – Pre OGL	0.092	0.686
Energy Expenditure (kJ.kgLBM^{-1}.min^{-1}) – Post OGL	0.239	0.159
Carbohydrate Oxidation (g.kgLBM^{-1}.min^{-1}) – Pre OGL	0.008	0.600
Carbohydrate Oxidation (g.kgLBM^{-1}.min^{-1}) – Post OGL	0.098	0.792
Fat Oxidation (g.kgLBM^{-1}.min^{-1}) – Pre OGL	0.052	0.343
Fat Oxidation (g.kgLBM^{-1}.min^{-1}) – Post OGL	0.009	0.638

Table S-4.3 Analysis of covariance (ANCOVA) data with BMI and gender examined as covariates. FTO genotype allele as the fixed factor and outcomes (metabolic flexibility, energy expenditure and substrate oxidation) as the dependent factors.

	Partial eta-squared	Observed Power	*Power (1-beta)*
Genotype			
RER	0.020	0.296	0.704
Energy Expenditure (kJ.kgLBM^{-1}.min^{-1})	0.006	0.112	0.888
Carbohydrate Oxidation (g.kgLBM^{-1}.min^{-1})	0.007	0.124	0.876
Fat Oxidation (g.kgLBM^{-1}.min^{-1})	0.005	0.107	0.893
BMI			
RER	0.001	0.067	0.933
Energy Expenditure (kJ.kgLBM^{-1}.min^{-1})	0.004	0.106	0.894
Carbohydrate Oxidation (g.kgLBM^{-1}.min^{-1})	0.060	0.831	0.169
Fat Oxidation (g.kgLBM^{-1}.min^{-1})	0.056	0.803	0.197

Table S-4.4 Power analysis of respiratory gas measurements. Partial eta-squared (effect size), *p* value (effect significance) and sample size (n) are used to determine the observed power (i.e. for RER between genotypes there is a 29.6% chance of detecting a true difference). Power (1-beta) reflects the probability of a type II error (i.e. for RER between genotypes there is a 70.4% probability of a type II error).

Gender	Male	Female	*P value*
n	13	15	
Age (yr)	27.5 ± 1.6	23.7 ± 1.4	0.090
Total Body Mass (kg)	79.0 ± 2.2	68.1 ± 2.7	**0.005**
Height (cm)	176.8 ± 2.1	169.3 ± 1.6	**0.007**
BMI (kg/m^2)	25.3 ± 0.7	23.7 ± 0.9	0.204
Hip Circumference (cm)	100.7 ± 1.5	99.7 ± 2.0	0.698
Waist Circumference (cm)	83.4 ± 1.7	76.7 ± 2.9	0.062
Fat Mass (%)	18.8 ± 1.2	30.1 ± 1.2	**<0.001**
Fat Mass (kg)	14.8 ± 1.2	20.7 ± 1.5	**0.007**
Lean Muscle Mass (kg)	60.1 ± 1.5	44.7 ± 1.3	**<0.001**
Bone Mineral Content (kg)	2.7 ± 0.1	2.4 ± 0.1	**0.015**
Systolic BP (mmHg)	132.0 ± 3.8	122.4 ± 2.4	**0.037**
Diastolic BP (mmHg)	75.2 ± 2.5	76.3 ± 2.5	0.759
Fasting Plasma Glucose ($mmol.L^{-1}$)	5.2 ± 0.1	5.0 ± 0.1	0.249
VO_{2peak} ($ml.kg.min^{-1}$)	42.2 ± 1.3	36.3 ± 2.6	**0.010**
80% VO_{2peak} Workload (W)	150.5 ± 10.9	98.5 ± 7.6	**<0.001**
40% VO_{2peak} Workload (W)	75.3 ± 5.4	49.3 ± 7.5	**<0.001**

Table S-5.1 Participant characteristics when separated by gender. Values are expressed as mean ± SEM. Total body mass, height, muscle mass, bone mineral content, systolic BP, VO_{2peak}, and 80% and 40% VO_{2peak} workloads were significantly higher in males compared to females (bolded, $p < 0.05$). Males had significantly lower fat mass than females (bolded, $p < 0.05$).

	Genotype			
Males (n=13)	**AA**	**AT**	**TT**	***P value***
n	5	5	3	
Age (yr)	26.4 ± 2.9	29.8 ± 1.0	25.3 ± 3.4	0.557
Total Body Mass (kg)	84.7 ± 2.3	72.0 ± 2.1	80.8 ± 3.8	**0.022**
Height (cm)	181.6 ± 3.9	173.0 ± 1.5	175.2 ± 1.5	0.294
BMI (kg/m^2)	25.9 ± 1.5	24.0 ± 0.5	26.4 ± 1.7	0.400
Hip Circumference (cm)	104.5 ± 1.9	96.3 ± 1.2	101.8 ± 2.3	**0.035**
Waist Circumference (cm)	86.7 ± 2.7	78.8 ± 1.5	85.7 ± 3.1	0.070
Fat Mass (kg)	17.6 ± 1.9	10.6 ± 0.8	17.1 ± 1.0	**0.009**
Lean Muscle Mass (kg)	62.8 ± 2.6	57.7 ± 2.4	59.5 ± 2.4	0.352
Bone Mineral Content (kg)	2.8 ± 0.2	2.5 ± 0.1	2.8 ± 0.2	0.255
Systolic BP (mmHg)	139.6 ± 5.9	129.2 ± 7.3	124.0 ± 0.6	0.273
Diastolic BP (mmHg)	76.6 ± 3.2	74.8 ± 4.1	73.7 ± 8.1	0.910
Fasting Plasma Glucose ($mmol.L^{-1}$)	5.1 ± 0.1	5.4 ± 0.2	5.0 ± 0.4	0.622
VO_{2peak} ($ml.kg.min^{-1}$)	40.7 ± 2.1	42.1 ± 1.7	44.7 ± 3.5	0.532
80% VO_{2peak} Workload (W)	144.6 ± 23.2	149.8 ± 12.3	161.7 ± 26.0	0.859
40% VO_{2peak} Workload (W)	72.4 ± 11.5	74.8 ± 6.1	81.0 ± 13.1	0.856
Females (n=15)	**AA**	**AT**	**TT**	
n	5	4	3	
Age (yr)	22.4 ± 1.5	28.8 ± 4.3	21.3 ± 0.6	0.079
Total Body Mass (kg)	64.3 ± 3.2	72.0 ± 4.9	68.6 ± 5.4	0.576
Height (cm)	170.5 ± 2.3	166.1 ± 3.6	170.5 ± 2.6	0.504
BMI (kg/m^2)	22.1 ± 0.7	26.2 ± 2.4	23.4 ± 1.4	0.219
Hip Circumference (cm)	94.8 ± 3.0	103.7 ± 4.1	101.2 ± 2.7	0.182
Waist Circumference (cm)	76.9 ± 6.8	80.8 ± 5.4	73.8 ± 3.3	0.658
Fat Mass (kg)	18.6 ± 0.8	23.0 ± 3.7	20.8 ± 2.9	0.561
Lean Muscle Mass (kg)	44.0 ± 1.7	45.5 ± 2.5	44.7 ± 2.8	0.923
Bone Mineral Content (kg)	2.3 ± 0.2	2.4 ± 0.2	2.3 ± 0.1	0.906
Systolic BP (mmHg)	118.4 ± 5.1	128.0 ± 4.1	122.0 ± 3.0	0.315
Diastolic BP (mmHg)	76.2 ± 6.1	77.0 ± 2.9	75.8 ± 2.7	0.982
Fasting Plasma Glucose ($mmol.L^{-1}$)	4.9 ± 0.1	5.4 ± 0.2	4.8 ± 0.1	**0.043**
VO_{2peak} ($ml.kg.min^{-1}$)	39.3 ± 1.9	35.2 ± 5.2	34.4 ± 1.8	0.446
80% VO_{2peak} Workload (W)	110.6 ± 9.7	96.3 ± 23.7	90.0 ± 8.7	0.538
40% VO_{2peak} Workload (W)	55.2 ± 5.0	48.0 ± 11.8	45.2 ± 4.4	0.553

Table S-5.2 Participant characteristics based on their FTO rs9939609 genotype and separated for gender. Values are expressed as mean ± SEM. Within male participants, a genotype effect was detected for total body mass, hip circumference and fat mass (bolded, $p < 0.05$). Subsequent analysis showed male AA genotypes to have a significantly greater total body mass ($p = 0.009$), hip circumference ($p = 0.017$) and fat mass ($p = 0.010$) compared to male AT genotypes. Male TT genotypes also had significantly greater fat mass compared to male AT genotypes ($p = 0.003$). Similar participant characteristics were observed between male AA and TT genotypes. Within female participants, a genotype effect was detected for fasting blood glucose ($p = 0.043$), with female AT genotypes having a higher concentration than female AA genotypes ($p = 0.047$) and female TT genotypes ($p = 0.023$). Similar participant characteristics were observed between female AA and TT genotypes.

Metabolism of other Co-Factors
Pyrophosphate
AMP
ATP
(CHO, Lipid & Purine Metab.)
GABA
GHB
Ethylphosphate
Beta-alanine
Vit B5
AA
AT
TT
Lipid Metabolism
Saturated Fatty Acids
Octadecanoate
Hexadecanoate
Nonanoate
Glycerolipid Metabolism
Monooctadecan.
Monohexadecan.
Monopalmit.
Cholesterol
Myoinositol-1-P
Myoinositol
Purine & Nucleotide Metabolism
Adenosine
Uric Acid
Carbohydrate Metabolism
Mannose
Fructose
Glucose
Glucose-6-P
Fructose-6-P
Fructose-1,6-diP
Glycerate-3-P
Glycine
Serine
Tyrosine
Lactate
Alanine
PEP
Pyruvate
Acetyl-CoA
Citrate
Isocitrate
2-Oxoglutarate
Maltose
Glycerol
Maltitol
Glucose-1-P
Phenylalanine
Isoleucine
Leucine
Glyoxylate
Oxalate
Glycolate
Succinate
Fumarate
Malate
Oxaloacetate
Glycerol-3-P
Galactose
Ascorbate
Erythronate
Malonate
Threonine
Aspartate
Pyroglutamate
Glutamate
Proline
Ornithine
Urea
Nicotinamide

Figure S-5.1 Network analysis was used to leverage a system-wide perspective of the metabolomic data when investigating the relationship between genotypes and metabolic responses to HI and LO intensity exercise. This network represents metabolic responses to the HI intensity exercise (80% VO_{2peak}) protocol, with metabolites expressed as mean ± SEM for pre exercise (0 mins), and 10 and 90 mins passive recovery respectively as bars move from left to right. Metabolites are not to scale between each node. Red box fill represents the metabolites with a time*genotype interaction ($p < 0.05$); Blue box outline represents metabolites with a main effect for time interaction ($p < 0.05$).

Known Metabolite	Derivative	HMDB ID	Identification Ion	RT (min)	MSI (level)
Adenosine	4TMS	HMDB00050	245	19.434	1
Alanine	2TMS	HMDB00161	190	8.547	1
AMP	5TMS	HMDB00045	382	21.450	1
Aspartate	3TMS	HMDB00191	232	12.303	1
Beta-alanine	2TMS	HMDB00056	218	9.391	1
Cholesterol	1TMS	HMDB00067	458	20.048	1
Citrate	4TMS	HMDB00094	273	14.535	1
Erythronate	4TMS	HMDB00613	292	12.554	1
Ethylphosphate	2TMS	HMDB12228	256	9.252	1
Fumarate	2TMS	HMDB00134	245	10.846	1
GABA	2TMS	HMDB00112	204	9.191	1
GHB	2TMS	HMDB15507	233	9.073	1
Glucose	1MEOX/5TMS	HMDB00122	319	15.191	1
Glucose-6-P	1MEOX/6TMS	HMDB01401	387	17.665	1
Glutamate	3TMS	HMDB03339	348	13.090	1
Glycerate-3-P	4TMS	HMDB00807	357	14.447	1
Glycerol-3-P	4TMS	HMDB00126	299	14.129	1
Glycine	2TMS	HMDB00123	204	8.731	1
Glycolate	2TMS	HMDB00115	205	8.298	1
Hexadeconate	1TMS	HMDB00220	313	16.134	1
Isoleucine	2TMS	HMDB00172	218	10.323	1
Lactate	2TMS	HMDB00190	191	8.149	1
Leucine	2TMS	HMDB00687	158	10.113	1
Malate	2TMS	HMDB00744	232	10.118	1
Malonate	2TMS	HMDB00691	481	15.354	1
Maltitol	9TMS	HMDB02928	531	20.300	1
Maltose	1MEOX/8TMS	HMDB00163	361	19.842	1
Mannose	1MEOX/5TMS	HMDB00169	319	15.051	1
Monohexadecanoylglycerol	2TMS	HMDB31074	371	19.172	1
Monooctadecanoylglycerol	2TMS	HMDB31075	399	20.120	1
Monopalmitoylglycerol	2TMS	HMDB31074	313	18.989	1
Myoinositol	6TMS	HMDB00211	432	16.357	1
Myoinositol-1-P	7TMS	HMDB00213	318	18.218	1
Nicotinamide	1TMS	HMDB01406	179	12.154	1
Nonanoate	1TMS	HMDB00847	215	10.967	1
Octadecanoate	1TMS	HMDB00827	356	17.334	1
Oxalate	2TMS	HMDB02329	147	8.810	1
Pantothenate (Vit B5)	3TMS	HMDB00210	420	15.751	1
Phenylalanine	2TMS	HMDB00159	266	13.206	1
Proline	2TMS	HMDB00162	142	10.405	1
Pyroglutamate	2TMS	HMDB00267	156	12.423	1
Pyrophosphate	4TMS	HMDB00250	451	13.416	1
Serine	3TMS	HMDB00187	204	10.893	1
Succinate	2TMS	HMDB00254	247	10.528	1
Threonine	3TMS	HMDB00167	291	11.123	1
Tyrosine	3TMS	HMDB00158	280	15.420	1
Urea	2TMS	HMDB00294	189	10.060	1
Uric Acid	4TMS	HMDB00289	456	16.416	1

Table S-5.3 Parameters of the 48 metabolites identified within the initially investigated PBQC (verified within all samples). RT, retention time; MSI, metabolomics standards initiative.

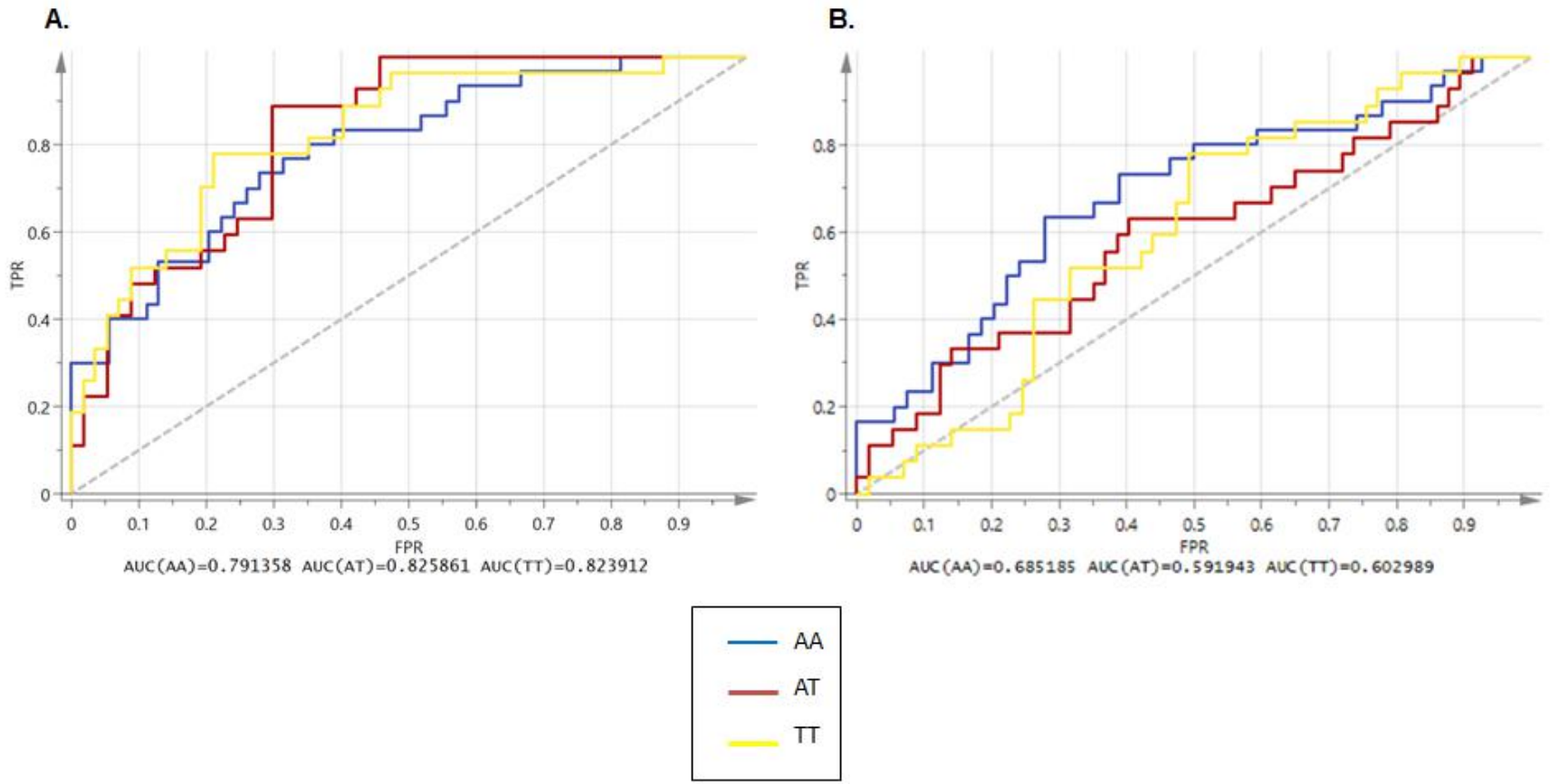


Figure S-5.2 AUC of the ROC curve for each O2PLS-DA model, A) HI and B) LO intensity exercise. The larger the AUC, the better is overall performance of the experiment to differentiate between genotypes, with 1.0 being the maximum. Similar AUC's in HI represents similar overall performance of genotypes to this exercise intensity. The ROC shows a poorer fit (closer to the diagonal) in the LO intensity exercise trial compared to HI intensity exercise trial, with AA genotypes better described by the model than TT genotypes. TPR, true positivity role (sensitivity); TNR, true negativity role (specificity).

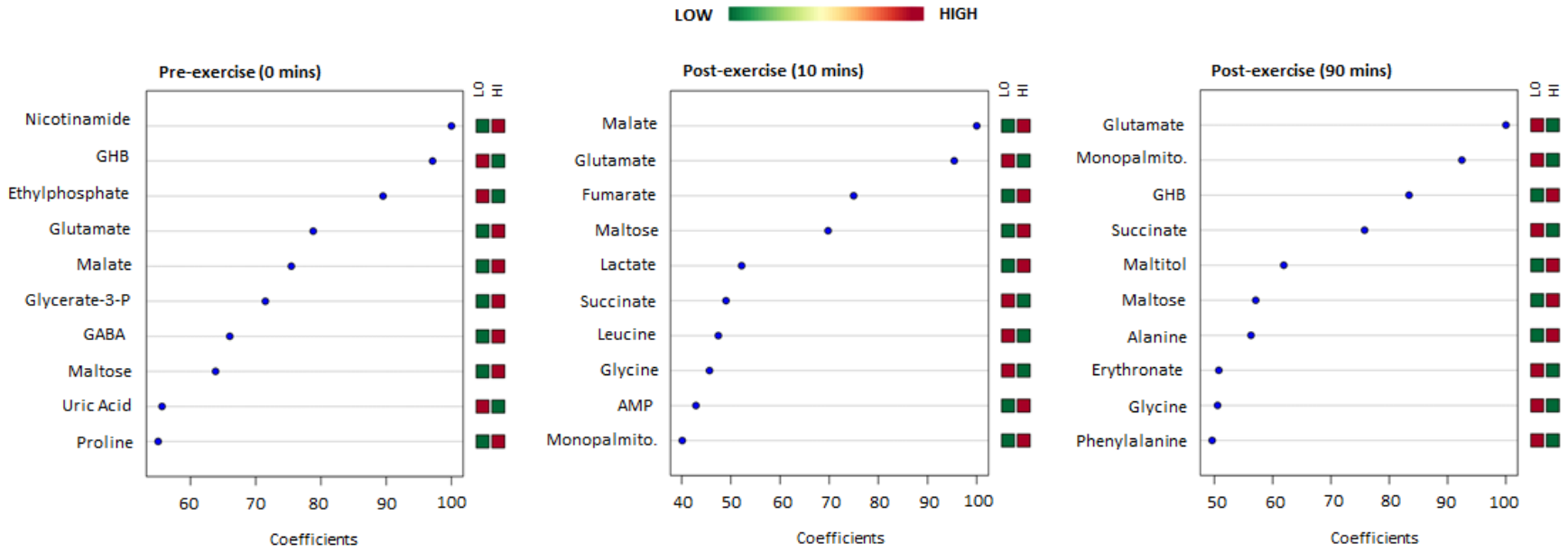


Figure S-5.3 The top 10 important features identified by overall coefficient scores of PLS-DA. Boxes of the right of each time points coefficient graph indicate the relative concentration of the corresponding metabolite in LO (40% VO_{2peak}) and HI (80% VO_{2peak}) intensity exercise. AMP, Adenosine-5-Monophosphate; GHB, Gamma Hydroxybutyric Acid; GABA, Gamma Aminobutyric Acid.

Metabolite	REST	REC10	REC90
Adenosine			
Alanine			1.44
AMP			
Aspartate			
Beta-alanine	1.58		
Cholesterol			1.06
Citrate			
Erythronate			1.23
Ethylphosphate	1.88		
Fumarate		2.30	
GABA	1.78		
GHB	2.11		2.16
Glucose	1.31	1.65	
Glucose-6-P			
Glutamate	1.76	2.18	2.30
Glycerate-3-P			1.30
Glycerol-3-P			
Glycine			1.31
Glycolate		1.50	
Hexadeconate			
Isoleucine			
Lactate		2.26	
Leucine			1.22
Malate	1.71	2.83	
Malonate			
Maltitol			1.50
Maltose	1.24	2.21	1.24
Mannose	1.39	1.97	
Monohexadecanoylglycerol			
Monooctadecanoylglycerol			
Monopalmitoylglycerol			2.20
Myoinositol			
Myoinositol-1-P			
Nicotinamide	2.17		
Nonanoate			
Octadecanoate			
Oxalate			
Pantothenate (Vit B5)			
Phenylalanine	1.29		1.28
Proline	1.49		
Pyroglutamate			
Pyrophosphate			1.00
Serine			
Succinate	1.08		1.77
Threonine			
Tyrosine			1.09
Urea			
Uric Acid	1.35		1.20

Table S-5.4 Metabolites with VIP scores of ≥ 1 for each time point during as determined by PLS-DA.

VIP Metabolite	HI			LO		
	Time	Genotype	Genotype x Time	Time	Genotype	Genotype x Time
Adenosine	< 0.001			0.001		
Alanine	0.002			0.001		
AMP						
ß-alanine						
Cholesterol						
Citrate						
Erythronate	0.004			0.008		
Ethylphosphate						
Fumarate	< 0.001			< 0.001		
GABA						
GHB	0.002					
Glucose	< 0.001		0.036	0.004		0.035
Glucose-6-P	0.001			0.018		
Glutamate	< 0.001			0.009		
Glycerate-3-P				0.004		
Glycerol-3-P						
Glycine	0.010					
Glycolate	< 0.001			< 0.001		
Hexadeconate						
Isoleucine	< 0.001					
Lactate	< 0.001			< 0.001		
Leucine	0.001					
Malate	< 0.001			< 0.001		
Malonate	< 0.001			0.013		
Maltitol						
Maltose	0.001			0.001		
Mannose	< 0.001					
Monohexadeca.						
Monooctadeca.	0.041					
Monopalmito.	0.032			0.034		
Myoinositol						
Myoinositol-1-P						
Nicotinamide	< 0.001					
Nonanoate						
Octadecanoate						
Oxalate				0.020		
Pantothenate				0.041		
Phenylalanine	< 0.001					
Proline	< 0.001					
Pyroglutamate						
Pyrophosphate				0.011		
Serine						
Succinate						
Threonine						
Tyrosine	0.001			0.011		
Urea	0.018					
Uric Acid	0.002					

Table S-5.5 Skeletal muscle metabolites in which a significant main effect for time, genotype main effect, or genotype by time interaction were determined (regardless of VIP value). $p < 0.05$.

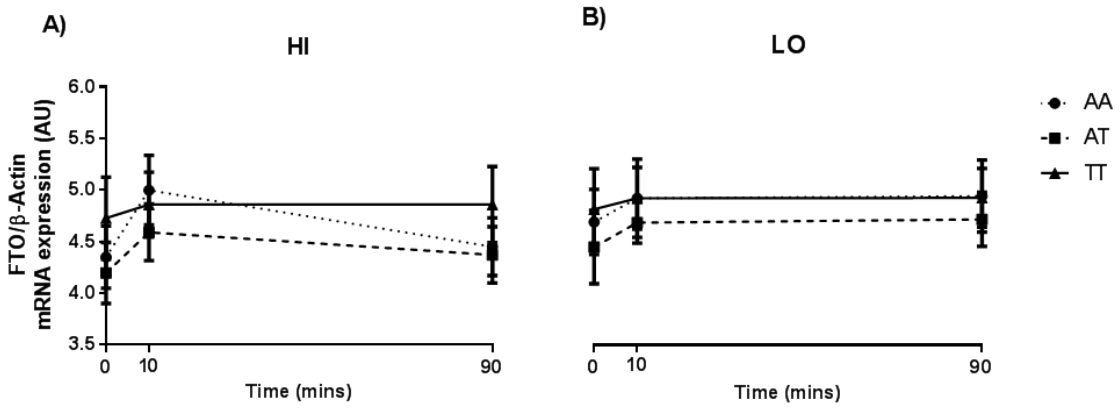


Figure S-6.1 Relative expression of FTO mRNA normalised to β-Actin in human vastus lateralis skeletal muscle presented as arbitrary units (AU). Data is expressed as mean ± SEM pre-exercise (0), and at 10 and 90 mins following A) 80% VO_{2peak} (HI) and B) 40% VO_{2peak} (LO) intensity exercise. A significant main effect for time was observed for FTO mRNA expression following high intensity exercise at 80% VO_{2peak} ($p < 0.001$). Subsequent pairwise comparisons revealed a significant increase in FTO mRNA expression from pre exercise to 10 mins post exercise ($p < 0.05$). No genotype main effect ($p = 0.804$), or genotype by time interaction ($p = 0.578$) were observed for high intensity exercise. No main effect for time ($p = 0.165$), genotype main effect ($p = 0.670$), or genotype by time interaction ($p = 0.853$) was identified for FTO mRNA expression following LO intensity exercise at 40% VO_{2peak}.

Known Metabolite	Derivative	HMDB ID	Identification Ion	RT (min)	MSI (level)
4-Hydroxyproline	3TMS	HMDB00725	230	12.540	1
Alanine	2TMS	HMDB00161	190	8.777	1
Arginine	3TMS	HMDB00517	256	14.810	1
Asparagine	3TMS	HMDB00168	231	13.684	1
Aspartate	2TMS	HMDB00191	306	12.479	1
Cholesterol	1TMS	HMDB00067	458	22.263	1
Citrate	2TMS	HMDB00094	465	14.734	1
Dodecanoate	1TMS	HMDB00638	257	13.584	1
Erythronate	2TMS	HMDB00613	292	12.627	1
Fructose	1MEOX/5TMS	HMDB00660	319	15.030	1
Fumarate	2TMS	HMDB00134	245	11.051	1
Glutamate	3TMS	HMDB03339	246	13.279	1
Gluconic Acid	6TMS	HMDB00625	333	15.948	1
Glucose	1MEOX/5TMS	HMDB00122	319	15.168	1
Glycine	2TMS	HMDB00123	204	8.953	1
Glycerol	3TMS	HMDB00131	205	10.301	1
Glycerol-3-P	4TMS	HMDB00126	299	14.317	1
Hexadecanoic Acid Methyl Ester	1TMS	HMDB61859	270	15.600	1
Hexadecanoate	1TMS	HMDB00220	313	16.338	1
Isoleucine	1TMS	HMDB00172	188	9.530	1
Inositol	6TMS	HMDB02985	432	16.547	1
Lactate	2TMS	HMDB00190	191	8.101	1
Leucine	2TMS	HMDB00687	158	10.325	1
Lysine	4TMS	HMDB00182	230	15.483	1
Malate	3TMS	HMDB00744	335	12.199	1
Mannose	1MEOX/5TMS	HMDB00169	319	15.395	1
Octadecanoate	1TMS	HMDB00827	356	17.532	1
Phenylalanine	2TMS	HMDB00159	192	13.411	1
Pipecolate	2TMS	HMDB00070	156	10.904	1
Proline	2TMS	HMDB00162	186	13.056	1
Rhamnose	4TMS	HMDB00849	277	14.052	1
Serine	3TMS	HMDB00187	204	11.098	1
Sorbose	1MEOX/5TMS	HMDB01266	307	15.130	1
Succinate	2TMS	HMDB00254	247	10.731	1
Sucrose	8TMS	HMDB00258	451	19.512	1
Tartaric Acid	4TMS	HMDB00956	219	12.952	1
Threonine	3TMS	HMDB00167	218	11.323	1
Tyrosine	3TMS	HMDB00158	308	15.420	1
Urea	2TMS	HMDB00294	189	10.450	1
Uric Acid	4TMS	HMDB00289	456	16.621	1
Valine	2TMS	HMDB00883	144	9.822	1
Xylitol	5TMS	HMDB02917	307	13.979	1
Xylose	4TMS	HMDB00098	217	13.547	1

Table S-7.1 Parameters of the 43 known metabolites within the initially investigated PBQC (verified within all samples). RT, retention time; MSI, metabolomics standards initiative.

Unknown Metabolite	Identification Ion	RT (min)	REST x-FC	*p*	EX10 x-FC	*p*	EX30 x-FC	*p*	RC60 x-FC	*p*
1	258	8.91	-1.21 ± 0.07		-1.07 ± 0.04		1.11 ± 0.07		-1.413 ± 0.07	*<0.001*
2	233	9.98	1.16 ± 0.18		-1.29 ± 0.10		-1.40 ± 0.08	*0.020*	-1.426 ± 0.09	
3	246	12.15	1.41 ± 0.48		-1.19 ± 0.31		-1.72 ± 0.24		-2.293 ± 0.26	*0.023*
4	292	12.75	1.49 ± 0.44		1.04 ± 0.49		-1.59 ± 0.10	*0.017*	-2.077 ± 0.05	*0.014*
5	329	12.85	-1.21 ± 0.18		-1.85 ± 0.15	*0.001*	-3.03 ± 0.17	*0.001*	-4.206 ± 0.17	*<0.001*
6	313	12.86	1.36 ± 0.17		-1.17 ± 0.10		-2.10 ± 0.14	*0.033*	-1.958 ± 0.14	*0.001*
7	292	13.08	-1.01 ± 0.19		-1.66 ± 0.24		-2.21 ± 0.22	*0.035*	-3.009 ± 0.25	*0.004*
8	326	13.73	-1.35 ± 0.15		1.58 ± 0.14	*0.009*	1.84 ± 0.07	*0.001*	1.007 ± 0.19	
9	369	14.87	1.36 ± 0.11		-1.58 ± 0.05	*<0.001*	-2.72 ± 0.09	*0.001*	-1.524 ± 0.10	*0.004*
10	565	17.84	1.13 ± 0.11		-1.74 ± 0.14	*0.011*	-5.35 ± 0.18		-4.125 ± 0.09	*<0.001*
11	329	18.32	1.24 ± 0.32		-2.57 ± 0.39	*0.020*	-2.50 ± 0.26		-9.844 ± 0.37	
12	363	18.38	-1.11 ± 0.37		1.07 ± 0.35		-3.20 ± 0.23	*0.007*	-3.253 ± 0.23	*0.020*

Table S-7.2 Quantification ion, retention time (RT), x-FC (change in $HIE_{300\%}$ compared to the $HIE_{150\%}$ protocol) and *p* value for metabolites with unknown identities that showed significant differences between trials throughout the timeframe examined.

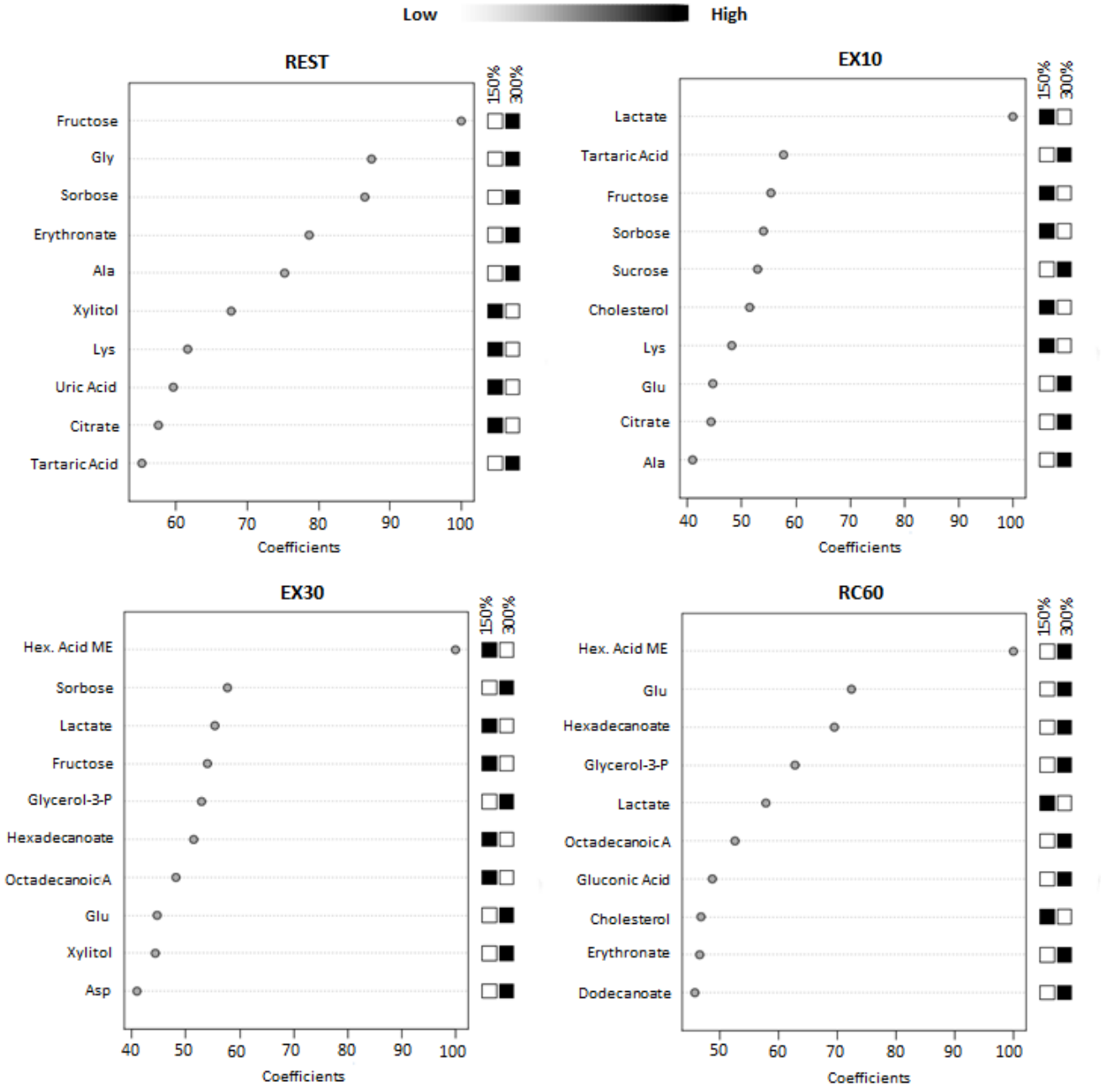


Figure S-7.1 Important features identified by overall coefficient scores of PLS-DA. Boxes on the right of each time points coefficient graph indicate the relative concentration of the corresponding metabolite in each exercise intensity performed.

Metabolite	REST	EX10	EX30	RC60
4-Hydroxyproline				
Alanine	1.76	1.21		
Arginine				
Asparagine	1.10		1.31	1.00
Aspartate		1.54		1.71
Cholesterol			1.00	1.23
Citrate	1.30	1.36	1.01	
Dodecanoate				1.27
Erythronate	2.10	1.79		1.22
Fructose	2.37	1.52	1.70	
Fumarate				
Glutamate	1.20	1.36	1.41	1.98
Gluconic Acid	1.51	1.38	1.12	1.28
Glucose				
Glycine	1.95			
Glycerol				
Glycerol-3-P	1.11	1.56	1.62	1.65
Hexadecanoic Acid Methyl Ester	1.28	1.42	3.14	2.62
Hexadecanoate			1.54	1.82
Isoleucine				
Inositol				
Lactate		2.76	1.78	1.55
Leucine				
Lysine	1.54	1.21		
Malate	1.08			
Mannose				
Octadecanoate			1.34	1.38
Phenylalanine				
Pipecolate				
Proline				
Rhamnose				
Serine				
Sorbose	2.00	1.43	1.87	
Succinate				1.00
Sucrose		1.47		
Tartaric Acid	1.56	2.12		1.09
Threonine				
Tyrosine				
Urea				
Uric Acid	1.44			1.00
Valine				
Xylitol	1.56		1.18	1.26
Xylose		1.39		1.08

Table S-7.3 Metabolites with VIP scores of ≥ 1 for each time point as determined by PLS-DA.